REMARKS

I. The Subject Matter of the Claims

In general, the subject matter of the claims relates to monoclonal antibodies specifically reactive with α_d integrin which also modulate TNF-α activity. The foregoing amendment is in the revised amendment format as provided in 1267 OG 106. Accordingly, the provisions of 37 C.F.R. § 1.21, requiring submission of clean and marked-up versions of the replacement paragraphs and claims, are waived.

II. The Objections to the Specification

Applicants note the Examiner's objections in paragraph 3 regarding the formal drawings and submit corrected drawings herewith.

The Examiner states in paragraph 4 that the title of the invention is not descriptive. Applicants have amended the title to read "Modulation of TNF-α with Integrin α_d-Specific Antibodies."

In response to paragraph 5 of the Office Action, the first paragraph of the specification has been amended to update the priority claim of the application and the status of the priority applications.

The Examiner states in paragraph 6 that all trademarks are to be indicated appropriately. Applicants submit that to the best of their knowledge all trademarks in the application are designated correctly.

The Examiner states in paragraph 7 that some sequence identifiers appear to be missing, *e.g.*, at page 125 of the specification. Applicants submit that due to the page breaks between pages 124-125 of the specification, the sequence identifier for the sequence at page 125, line 1, is found on the previous page at page 124, line 32. No other sequence identifiers are missing in the application.

III. Amendments

Support for the amendment to claim 11 and 12 can be found at page 5, lines 3-5, and at page 5, lines 22-24, which describe preferred α_d-encoding polynucleotides and α_d polypeptide molecules, respectively. Page 13, lines 26-28, describes homologous α_d molecules

encoded by polynucleotides which hybridize to polynucleotides encoding α_d and page 29, lines 29-32, describes hybridization conditions under which these polynucleotides will hybridize to other α_d-encoding polynucleotides.

Support for the amendment to claim 14 can be found at page 91, lines 18-27, which indicates that hybridoma fusion 205 was performed in a manner similar to fusion 199 in which the I-domain of the α_d protein was used as the antibody priming antigen (see page 88, lines 23-28). The amendment includes no new matter.

IV. Patentability Arguments

A. The Rejection of Claims 11-14 under 35 U.S.C. §112 First Paragraph, May Properly be Withdrawn

In paragraph 9 of the Office Action, the Examiner rejects claim 14 under 35 U.S.C. §112, first paragraph, for assertedly not being enabled by the specification. The Examiner alleges that the application did not enable claim 14 because it contained no indication of deposit information for the claimed hybridomas. Applicants submit that amendment to claim 14 to recite a specific antigen used to generate the antibodies, rather than hybridoma clone designation, obviates this rejection.

In paragraph 10 of the Office Action, the Examiner rejects claims 11-14 under 35 U.S.C. §112, first paragraph, as assertedly not being enabled by the specification for "any α_d specificity as the target of the claimed methods."

Applicants submit that amendment to claims 11 and 12 to recite specific α_d molecules obviates the Examiner's rejection. The amendment to the claims indicates that the anti-α_d monoclonal antibody is immunospecific for the α_d polypeptide encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 1, a polynucleotide that encodes the polypeptide of SEQ ID NO: 2; and a polynucleotide that hybridizes to the complement of either (i) the polynucleotide of SEQ ID NO: 1 or (ii) a polynucleotide that encodes a polypeptide of SEQ ID NO: 2. Support for the amendment is provided in Section III above.

B. The Rejection of Claims 11-14 under 35 U.S.C. §112, Second Paragraph, May Properly be Withdrawn

In paragraph 11.A, the Examiner asserts that claims 11-14 are indefinite in their recitation of the term "modulating." Applicants contend that "modulating" is clearly defined in the specification at page 19, line 8, to be either the inhibition or enhancement of a particular activity (in the instant case, TNF-α activity).

Applicants submit that because the specification specifically defines "modulation," there is no need to further define what type of modulation or what degree of modulation is necessary.

The Examiner asserts in Paragraph 11.B that claim 14 is indefinite in its recitation of hybridomas 205C and 205E because their characteristics are not known. Applicants submit that the amendment to claim 14 obviates this rejection.

C. The Rejection of Claims 11-13 under 35 U.S.C. §102(b), May Properly be Withdrawn.

In paragraph 13 the Examiner rejects claims 11-13 under 35 U.S.C. §102(b) for assertedly being anticipated by Gallatin, which allegedly teaches methods of treating immune or inflammatory responses with antibodies to α_d.

Applicants respectfully disagree. The present invention involves methods for specifically modulating TNF-α activity using monoclonal antibodies to α_d, and Gallatin does not disclose or suggest any ability of α_d-specific antibodies to modulate TNF activity. Gallatin simply describes a method for producing α_d-specific antibodies and discloses a general use for the disclosed antibodies, without giving <u>any</u> particular examples of α_d-specific monoclonal antibodies or methods for their use other than the generic use for treating immune or inflammatory responses. Applicants submit that methods of treating an immune response does not suggest, or even require, modulation of TNF-α activity. In addition, it is well-known in the art that inflammation can arise without involvement of TNF-α at all. See e.g., Feliciani *et al.,* "A Th2-like cytokine response is involved in bullous pemphigoid. The role of IL-4 and IL-5 in the pathogenesis of the disease" *Int J Immunopathol Pharmacol.* 12:55-61. 1999, and Takashi *et al.,* "Spontaneous B-cell IgE production in a patient with remarkable eosinophilia and hyper IgE" *Ann Allergy Asthma Immunol.* 2000 85:150-5 (References enclosed). As such, a worker of skill

in the art would not have realized the ability of these antibodies to modulate TNF-α activity until the present disclosure.

The Examiner contends that the mechanism of action has no bearing on an invention when that invention was already known or obvious. Anticipation, however, requires that a reference disclose each and every limitation of the claimed invention. Gallatin neither discloses nor suggests an antibody that functions as a modulator of TNF-α activity because that property of the α_d antibodies useful according to the invention was neither known nor obvious to a worker of skill at the time of filing. Thus, the Applicants submit that the rejection of claims 11-13 under 35 U.S.C. § 102(b) should properly be withdrawn.

D. The Rejection of Claims 11-14 under the doctrine of Obviousness-type Double Patenting May Properly be Withdrawn.

In paragraph 15 of the Office Action, the Examiner rejects claims 11-14 based on non-statutory obviousness-type double patenting as allegedly unpatentable over claims 1-10 of U.S. Patent No. 6,251,395 and claims 1-9 of U.S. Patent No. 6,432,404. The Examiner states that the pending claims directed to modulating TNF-α activity using α_d-specific antibodies would be an inherent property of the patented methods, which inhibit locomotor damage following spinal cord injury or inhibit inflammation in central nervous system (CNS) injury by administration of α_d antibodies.

Non-statutory obviousness-type double patenting requires comparison of the application claims and cited patent claims on a claim by claim basis. Use of the specification and all it discloses is improper except to establish a meaning for a claim term. See *In re Boylan* 392 F.2d 1017, 157 USPQ 370 (CCPA 1968). MPEP 804 states that non-statutory type double patenting is primarily intended to prohibit issuance of claims in a second patent not patentably distinguishable from claims in a first patent. A worker of skill in the art looking at the claims of either of U.S. Patent Nos. 6,251,395 or 6,432,404 would not realize that the subject matter of the claims would embrace the modulation of TNF-α activity. The patent claims recite methods for inhibiting macrophage infiltration or locomotor injury in the CNS using antibodies to α_d. Many activities facilitate the entry of macrophages into the CNS as well as modulate locomotor injury; e.g., Flugel *et al.*, (*Eur. J. Immunol.* 31:11-22. 2001) discloses that nerve growth factor can mediate macrophage entry in to the CNS, and Huang *et al.*, (*J. Exp. Med.* 193:713-26. 2001),

discloses that lack of the chemokine, macrophage chemotactic protein-1 (MCP-1) prevents macrophage infiltrate into the CNS (References enclosed). Administration of α_d-specific antibodies therefore could modulate macrophage infiltration in to the brain by a number of different mechanisms, and it would not be at all apparent to a worker of skill in the art that the patented claims would embrace the modulation of TNF-α activity. Thus, the patent claims cannot render obvious methods of modulating TNF-α recited in the currently pending claims and the obviousness-type double patenting rejection should properly be withdrawn.
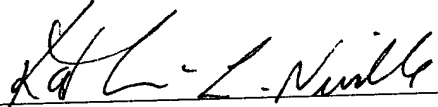
V. Conclusion

In view of the amendments and remarks made herein, Applicants submit that claims 11-14 are in condition for allowance and respectfully request expedited notification of the same.

Respectfully submitted,

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A TH2-LIKE CYTOKINE RESPONSE IS INVOLVED IN BULLOUS PEMPHIGOID. THE ROLE OF IL-4 AND IL-5 IN THE PATHOGENESIS OF THE DISEASE.

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Bullous Pemphigoid is an autoimmune bullous disorder characterized by production of IgG against an hemidesmosomal antigen (230 kDa, 180 kDa) responsible for blistering of the skin. In the past several mediators have been implicated in the pathogenesis of the disease such as proteases and collagenases secreted by local inflammatory cells. In order to investigate the role of cytokines in BP, the cytokine pattern was evaluated by an immunohistochemical analysis and by reverse transcriptase polymerase chain reaction procedure in 13 BP patients. Cytokines examined were interleukin (IL)-2, IL-4, IL-5, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The T cell inflammatory infiltrate was also characterized by monoclonal antibodies showing CD3+, CD4+ T cells with a perivascular and scattered distribution in lesional skin. IL-4 and IL-5 were detected in a similar distribution to the inflammatory infiltrate. IL-4 and IL-5 mRNA levels were also revealed by RT-PCR. Proinflammatory cytokines such as TNF-α, IL-6 and Th1-like cytokines (IL-2 and INF-γ) were not detected neither as proteins nor as mRNA. Since IL-4 and IL-5 are important in eosinophil chemoattraction, maturation and functional activity, the presence of IL-4 and IL-5 in BP suggest that these cytokines could be important in the pathogenesis of the disease.

Bullous Pemphigoid (BP) is a subepidermal blistering disorder characterized by linear depositions of complement and IgG autoantibodies against the basement membrane zone (BMZ). Histological and immunohistological examination of BP lesions show T cells (mainly CD4+), neutrophils, eosinophils and mastcells (1-3). CD4+ T cells are divided into T helper1 (Th1) and a Th2 phenotypes. The Th1 phenotype produces IL-2, TNF-β and IFN-γ important in delayed hypersensitivity, the Th2 phenotype, which provides support to the humoral response, synthetizes IL-4, IL-5, IL-6, IL-10, IL13, and GM-CSF (4-6). Th2-like cytokines are important mediators in eosinophil activity, growth and maturation, they have been implicated in diseases where eosinophilia is prominent such as asthma (7) and atopic dermatitis

(8). Studies involved in the comprehension and evaluation of the blistering process, in the disease, have progressively outlined the role of the inflammatory infiltrate (9-10). There are reports showing a Th1-like involvement in BP (11-13). Significant high levels of IFN-γ were found in serum and blister fluid of BP patients (11-13). IFN-γ has also been demonstrated to induce dermal-epidermal split, within the lamina lucida, in explanted cultured human skin (13). Elevated levels of IL-2R (14), TNF-α (15), TNF-β (16) and an IL-1 and IL-2 like activity (17) have been demonstrated in BP blister fluid or in the sera of BP patients. On the other hand several authors have shown a Th2-like involvement in the pathogenesis of the disease suggesting a potential role of IL-4, IL-6, IL-10 and IL-5 documented in blister fluid of BP patients

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(18-19). IL-4 and IL-5 are important cytokines in eosinophil chemoattraction and maturation (20-21). Eosinophils have assumed an important role in the pathogenesis of several skin diseases, including autoimmune bullous disorders (22). Eosinophil derived proteins, presumably proteases, seem to be directly involved in the dermal epidermal split (23) .

A prevalent Th2 type phenotype in other chronic subepidermal bullous diseases such as linear IgA dermatosis (24) and dermatitis herpetiformis (25) have been recently reported. This is the first report showing a Th2-like cytokine expression at a molecular level in BP and we suppose that IL-4 and IL-5 are important mediators in the development of the disease.

MATERIALS AND METHODS

Patients

Thirteen patients affected by BP were selected for this study (7 male, 6 female). Age ranged from 48 to 81 years (median age 65.8). Diagnosis was established according to clinical, histopathologic and immunologic (direct and indirect immunofluorescence on suction split-skin) criteria. All specimens were from patients with a recent onset of the disease and without any previous specific treatment. Skin specimens were immediately frozen in liquid nitrogen and stored at -80°C until use.

Immunohistochemistry

Cryostat sections were cut at 6(m and air dried for 30 minutes without fixation. Endogenous peroxidase was quenched in 0.3% H_2O_2. Single immunolabelling on serial sections was performed as previously described (26) using monoclonal mouse anti-human antibodies CD3, CD4, CD8, (YLEM, Rome, Italy), CD45RO (Dako, Glostrup, Denmark), TNF-α, IL-2, IL-4, IL-6, and IFN-γ; rabbit anti-human IL-5, IL-8 (Genzyme, Cambridge, USA), and TNF-α (Pepro-Tech, Rocky Hill, NJ, USA). Skin sections were incubated with primary antibodies, then incubated with biotinylated rabbit anti-mouse IgG or goat anti rabbit and finally reacted with HRP-streptavidin. In brief, primary antibodies were incubated at room temperature for 60-90 min in a moist chamber. For immunoperoxidase staining we used a streptoavidin-biotin system kit for primary mouse and rabbit antibodies (YLEM, Avezzano, Italy).

Diaminobenzidine tetrahydrochloride in PBS containing 0.01% fresh hydrogen peroxide was used as a chromogenic substrate. Sections were countstained with hematoxylin, dehydrated and permanently mounted with Eukitt (Kindler GmbH & co., Freiburg, Germany) under a coverslip.

Negative controls were performed using 1) normal skin, 2) omitting the incubation with primary antibodies. Cytokine expression were assessed as follows: strong staining, ++++/+++; moderate staining, ++; weak/focal staining, +; the epitelial staining was described as positive or negative.

mRNA extraction and cDNA synthesis

mRNA was extracted using a purification system kit (Pharmacia Biotech, Milan, Italy). In brief, about 30 of 20 μm cryostat sections from a 6 mm punch biopsy (about 0.5 gr of frozen tissue) were dissolved in a solution containing guanidinium thiocyanate 4M and N-lauroylsarcosine in order to preserve the RNA. The solution was placed in oligo(dt)-cellulose at 25 mg/ml suspended in a storage buffer containing 0.15% Kathon CG (Pharmacia LKB, Cologno Monzese (MI), Italy). After several washes in salt buffers containing 10 mM Tris-HCl (PH 7.4), 1 mM EDTA, 0.5 M NaCl or 0.1 M NaCl in the last 2 washes, the oligo(dt)-cellulose containing mRNA was placed in filter columns and the mRNA was eluted in warm TRIS-HCl 10 mM and precipitated in chilled 95% ethanol overnight. After centrifugation, the pellet was dissolved in 14μl of DEPC-treated sterile water and quantitated by spectrophotometric analysis. 0.5 μg of mRNA was transcribed in cDNA incubating it with 200U of superscript reverse transcriptase (GIBCO BRL, Milan, Italy) and 50 ng of Random Examers.

Reverse Transcriptase-PCR

cDNA was amplified with 2.5 U Taq polymerase (Perkin Elmer Cetus, Milan, Italy) using 1.5 pM of each primer specific for IL-2, IL-4, IL-5, IFN-γ, β-actin. Primers were prepared from published sequences (27) using a DNA synthetizer (Applied Biosystem, Warrington, Great Britain). The sequences of specific primer pairs used in the PCR were as follows:

IL-2: upstream, 5'-ACTCACCAGGATGCTCACAT-3'; downstream 5'-AGGTAATCCATCTGTTCAGA-3'

IL-4: upstream, 5'-CTTCCCCCTCTGTTCTTCCT-3'; downstream, 5'-TTCCTGTCGAGCCGTTTCCAG-3'

IL-5: upstream, 5'-ATGAGGATGCTTCTGCATTTG-3'; downstream, 5'-TCAACTTTCTATTATCCACTCGGTGTTCATTAC-3'

IFN-γ: upstream 5'AGTTATATCTTGGCTTTTCA-3'; downstream, 5'-ACCGAATAATTAGTCAGCTT-3'

β-Actin: upstream, 5 '-GTGGGGCGCCCCAGG CAC-CA-3';
downstream, 5 '-CTCCTTAATGTCACGCACG ATTTC-3'

Each sample was divided in half, one part was used for the cytokine of interest, the other half for the house-keeping gene β-actin for semiquantitative analysis. RT-PCR was conducted for IL-4, IL-5, IL-2, IFN-γ and β-actin with the following protocol: 1 min. at 94°C, 1 min. at 58°C (62°C for IL-4 and β-actin), 1 min. at 72°. 30 cycles were used for cytokines of interest and 25 cycles for β-actin. The linear range of signal strength for each cytokine mRNA was determined by performing titration for cDNA and cycle numbers as previously described to obtain non saturated PCR reactions for each cytokine (28-29). Five μl of amplified products were electrophoretically separated in 2% agarose gel containing ethidium bromide and finally analyzed for molecular size. The following controls were used: 1) genomic DNA; 2) PCR products without primers; 3) normal skin (5 specimens). Signals were analysed by software Bio-profile (Vilber Lourmat, Nice, France), and semi-quantitative analysis was possible comparing the amplified product signals with the β-actin signal.

RESULTS

Clinical and immunophatological findings of BP patients are summarized in table 1. All patients demostrated depositions of IgG and C3 in the basement membrane and positive indirect immunofluorescence.

Table I also summarizes the immunohistochemical results and mRNA expression for each patient.

Immunohistochemistry

Immunohistochemical analysis showed a perivascular and scattered distribution of CD3+ T cells with a prevalent CD4+ T helper pattern with about 50% of CD45RO+ cells. CD45RA subset was also focally present.

Ten out of 13 patients affected by BP showed a positivity to IL-5 and IL-4. IL-4 and IL-5 staining showed a scattered and perivascular pattern in lesional (fig. 1a, 1c) and perilesional sites (fig.1b). Cells were mostly stained with an intracellular pattern, but some cells showed an extracellular staining. Rarely some intraepidermal cells were positive for IL-5 in BP (fig.1c). Controls and normal skin were negative. In lesional areas inflammatory cells consisting of CD4+ cell and

Case N.	Sex	Age	DIF	IIF	Cytokines staining	mRNA expression
BP1	M	75	IgG, C3	1:640	IL-4(+++), IL-5 (+++)	IL-4, IL-5
BP2	M	61	IgG, C3	1:320	IL-4(+++), IL-5(+++)	IL-4, IL-5
BP3	M	59	IgG, C3	1:160	IL-4(+), IL-5 (++)	IL-4, IL-5 (weak)
BP4	F	48	IgG, C3	1:320	IL-4(weak), IL-5(+++)	IL-4 (weak), IL-5
BP5	M	69	IgG, C3	1:160	-	IL-4, IL-5
BP6	M	50	IgG, C3	1:160	IL-4(+), IL-5(+++)	IL-4, IL-5
BP7	F	75	IgG, C3	1:160	IL-4(++), IL-5(+)	IL-4 (weak), IL-5
BP8	F	81	IgG, C3	1:160	-	IL-4, IL-5 (weak)
BP9	M	76	IgG, C3	1:320	IL-4(+), IL-5(+++)	IL-4 (weak), IL-5
BP10	F	67	IgG, C3	1:160	-	IL-4, IL-5
BP11	M	71	IgG, C3	1:640	IL-4(+++), IL-5(+++)	IL-4, IL-5
BP12	F	55	IgG, C3	1:320	IL-4(++), IL-5(+)	IL-4, IL-5 (weak)
BP13	F	69	IgG, C3	1:320	IL-4(+), IL-5(+++)	IL-4, IL-5

Table I. *Clinical and immunological characteristics of patients with BP:*
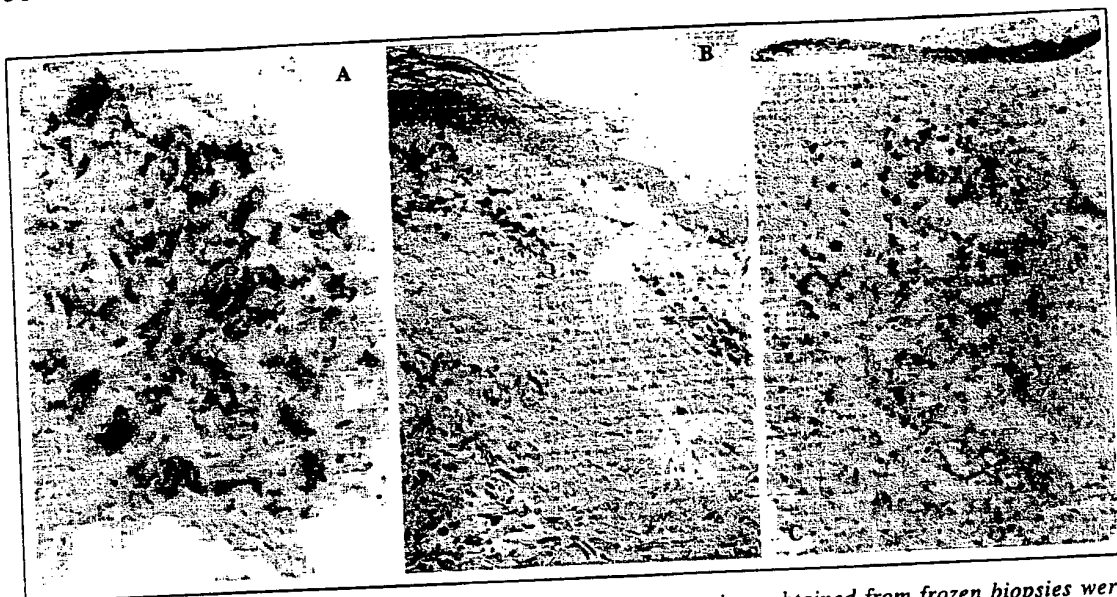*BP: Bullous Pemphigoid; DIF: Direct Immunofluorescence; IIF: Indirect immunofluorescence;   -: Negative*

Fig. 1. *Immunohistochemical detection of IL-4 and IL-5 in BP: sections obtained from frozen biopsies were stained by the streptoavidin-biotin complex immunoperoxidase method (streptoABC-system) as described in Materials and Methods. A similar distribution of both proteins was seen. a) Presence of IL-4 protein in a blister lesionf of BP; b) pattern of IL-4 staining in perilesional skin; c) IL-5 protein in lesional skin BP, few positive cells are present in the epidermis.*
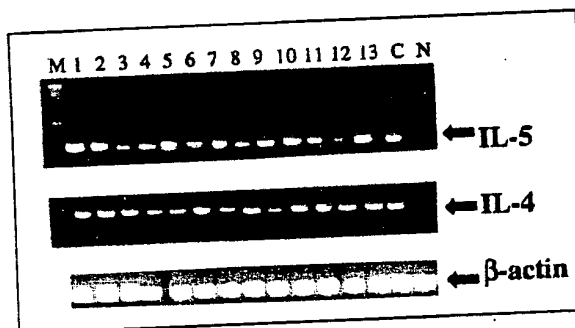

Fig. 2. *RT-PCR for IL-4 and IL-5 mRNA in Bullous Pemphigoid.*
Patients are numbered from 1 to 13. M=Marker; C= Positive Control; N= Normal skin.

eosinophils roughly corrisponded to the IL-4 and IL-5 staining. IFN-γ, IL-2 and TNF-α were under detectable levels.

Th2-like cytokines mRNA expression

As shown in figure 2 all BP patients expressed IL-4 and IL-5 mRNA. IL-4 or IL-5 expression appears not to be related to the disease activity. Most of the patients showed a similar expression between IL-4 and IL-5 (eg patients 1,2,4,10,11,13), others showed an higher IL-4 expression (eg. patients 3,6,8,12) and only two (patients 5 and 7) showed an higher IL-5 expression. Controls failed to express both IL-4 or IL-5 even after 50 PCR-cycles. No signals were detected for IL-2 after 60 cycles, IFN-γ was evident after more than 50 cycles both in normal and diseased skin in accordance with previously published results (27).

DISCUSSION

Bullous pemphigoid (BP) is an autoimmune blistering disease, characterized by linear IgG (mainly IgG4) and C3 deposits at the dermal-epidermal basement membrane (BMZ). A pathogenic role for specific antibodies has been demonstrated both in vitro on skin esplants (30) and in vivo using a passive transfer model in mice (31). Neverthless the presence of activated T cells within BP lesions and in the peripheral blood of patients, accompanied by eosinophilia, high levels of eosinophilic cationic

protein (ECP) and IgE, indicates the involvement of a cell mediated immune reaction in the process of blistering (32,33, 7, 10).

The T-cell component in BP displays a predominant T Helper pattern (CD4:CD8=2:1), contrasting data, however, regard the Th1 vs Th2 polarization of this infiltrate (1,2,7,34). Kaneko et al. report the presence of Th1 lymphocytes producing IFN-((12). Consistent with this finding IFN-(has been shown to induce, in vitro, a dermal epidermal split within the lamina lucida (11,13). More recently, however, a Th2-like activity has been demonstrated in BP. The finding of increased serum CD30 levels, a specific activation marker of cells able to produce Th2 cytokines (35), in BP patients, seems to suggest that these cytokines play a role in the disease (36). In the same study, De Pita and coll. found a correlation between sCD30 and IL-4 serum levels (36). However, more direct evidence for a role of Th2 cytokines in the process of blistering comes from studies showing a higher level of these mediators in BP blister fluids than in corresponding serum samples (37-39). Previous reports found IL-4 and IL-5 in BP blister fluid by ELISA method (37-40). In the present study we confirm, by immunohistochemistry, the presence of IL-4 and IL-5 protein within BP lesions, and we demostrate IL-4 and IL-5 mRNA expression in BP lesions. Although double staining with anti IL-4 and anti CD4 or with anti IL-5 and CD4 was not feasible, it is very likely that IL-4 and IL-5 found in the skin could be produced and expressed by by CD4+ cells and eosinophils. Infact most of the cellular infiltrate in BP is represented by eosinophils, neutrophils and T cells, though mast cells play a role as well (1,3,9). The majority of this T cell infiltrate has the characteristics of the activation state (expression of HLA-DR antigens, production of IL-2, association with CD1a+ positive cells) (2,10,14). In our study the finding of high levels of IL-4 and IL-5 both messenger and protein and almost undetectable messenger for IFN-γ clearly indicates that T cells are polarized towards a TH2 phenotype, as we have already shown in HD, another autoimmune subepidermal disease with tissue eosinophilia (25). Since we describe a local production of IL-4 and IL-5 in the site of blistering we think that these cytokines could play a central role in this process. We speculate that the binding of the autoantibody to the target antigen activates the complement thus producing an inflammatory infiltrate. Chemoattracted Th2 cells, producing IL-4 and IL-5 could induce eosinophil activation. IL-4 exerts pleiotropic effects by acting on eosinophil recruitment in tissues (41). It promotes eosinophil transmigration from vascular endothelium by upregulating important adhesion molecules such as ICAM1 (42) and VCAM1 (43). This last one has been detected on vessels in blistering areas of BP in association with E-selectin and P-selectin (44). IL-5 shares its properties on eosinophils with IL-4 although IL-4 seems to act mainly on growth (21), while IL-5 has effects on eosinophil chemoattraction and activation in vitro and in vivo (45-48). Experimental data indicate that the accumulation of eosinophils within BP lesions is directly related to blistering process (44). Infact eosinophil granule proteins, though represented in all stages of BP lesions, are most marked in early erythematous and prebullous (urticarial) areas and is minimal in uninvolved skin (44). Furthermore it has been demonstrated, in vitro, that 92 KD gelatinase, a protease expressed only by eosinophils in BP lesions, has the ability to cleave the extracellular collagenous domain of recombinant 180 kDa BP autoantigen, thus contributing significantly to the dermal epidermal split in BP (23).

Taken together, these data provide further support to the hypothesis that the infiltrating T cells in BP are stimulated immunologically, showing a preferential polarization toward Th2-like phenotype. We also speculate that IL-4 and IL-5 could play a central role in recruiting, in situ, polymorphonuclear cells able to release factors capable of detaching the basal membrane. In BP patients an immunologic response might be present which involves not only the production of IgG autoantibodies directed against specific cutaneous antigens but also the activation of T cells, polarized into a Th2-type profile. Th2 cells, producing IL-4 and IL-5, could induce eosinophil activation. Eosinophils, together with mast cells and neutrophils, in this environment, could produce proteases responsible for the proteolytic action on the basement membrane.

In conclusion our report supports the hypothesis that Th2-like cytokines IL-4 and IL-5, in association with other mediators, are important in mediating the local immune response in BP.

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Spontaneous B-cell IgE production in a patient with remarkable eosinophilia and hyper IgE

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Background: The pathophysiology of eosinophilia and hyper-IgE is not fully elucidated yet.

Objective: To clarify the pathophysiology of a patient with remarkable eosinophilia and hyper IgE, we examined cytokine levels in serum, surface antigens of peripheral blood eosinophils and IgE production in vitro.

Results: Concentrations of tumor necrosis factor-α (TNF-α), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), and granulocyte/macrophage-colony stimulating factor (GM-CSF) in the serum were 21 pg/mL, <15 pg/mL, <15 pg/mL, 8 pg/mL, and <5 pg/mL pg/mL, respectively. Newly expressed surface antigens CD4, CD25, CD69, and HLA-DR, but not CD54, were observed on peripheral blood eosinophils. Extremely high levels of IgE secretion was found in the patient's mononuclear cells without stimuli; this was not enhanced by IL-4 or IL-4 plus anti-CD40 monoclonal antibody stimulation. Furthermore, highly purified B cells spontaneously produced large amounts of IgE and the production was not enhanced in addition of his T cells.

Conclusion: The eosinophils were activated, and the B cells spontaneously produced IgE independently of T cells or cytokines, suggesting that intrinsic abnormality of B cells leading to disregulated production of IgE in this disease.

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INTRODUCTION

The IgE immune response is important because it leads to the development of allergic diseases such as rhinitis and bronchial asthma.[1] The production of immunoglobulin E (IgE) from B cells requires at least two signals: a class switching factor, most commonly interleukin (IL)-4, and the engagement of CD40 molecule on B cells. The combination of IL-4 and anti-CD40 monoclonal antibody (mAb) promotes IgE production in vitro.[2-4]

Eosinophilia is reported in various diseases such as bronchial asthma, parasite infection and Churg-Strauss syndrome. Mature eosinophils are developed in the presence of interleukin-3 (IL-3), interleukin-5 (IL-5), and granulocyte/macrophage-colony stimulating factor (GM-CSF).[5] The functions of eosinophils are survival elongation in vitro,[6] degranulation,[7] release of chemical mediator[5] and cytokine production.[8] Surface antigens on eosinophils are expressed by various cytokine stimulation[9] and the newly expressed surface antigens are suggested to be a hallmark of activation.[10,11]

To clarify the pathophysiology of a patient with high levels of serum IgE and remarkable eosinophilia, we have examined the concentration of cytokines in serum, surface analysis of eosinophils and IgE synthesis in vitro.

CASE REPORT

A 82-year-old man was transferred to our hospital because of edema on the extremities, systemic erythema, eosinophilia, and high levels of serum IgE. He was treated twice for tuberculous pleurisy (16 years old and 26 years old). He had suffered from atopic dermatitis for 5 years and was treated with steroid ointment. In August 1997, he was admitted to a hospital because of pyrexia. A diagnosis of pulmonary tuberculosis was made based on positive acid-fast stain of the sputum and consolidation shadow of right middle-lower lung fields on chest roentgenogram; the patient was administered the anti-tuberculous drugs isoniazid, rifampicin and ethambutol. Although liver dysfunction occurred (GOT 500 U/L, GPT 487 U/L, LDH 1,092 U/L), it was improved by quitting administration of the anti-tuberculous drugs. The number of peripheral blood eosinophils, however, gradually increased (2,000 to 8,000/μL), and systemic erythema worsened in conjunction with the appearance of edema in the lower extremities. On November 19, 1997 he was transferred to our hospital for further examination. His temperature was 37.6°C; several 5 to 10 mm sized superficial lymph nodes were palpable on the bilateral neck, left axilla and bilateral groins. Liver was palpable 3 cm on right midclavicular line. White blood cells of peripheral blood (15,700/μL), eosinophils (8,007/μL) and LDH (813 U/L) were elevated. Serum IgE and CRP were 25,207 IU/mL and 1.09 mg/dL, respectively. Drug-induced lymphocyte stimulation test for antituberculous drugs was negative. Repeated tests for parasites in the feces were negative. Chest roentgenogram showed consolidation on right middle-lower lung fields with pleural calcification. Biopsy and aspiration of bone marrow showed increased cell number of mature eosinophils without malignant cells and a lymph node biopsy specimen taken from the right inguinal region showed no malignancy. Steroid ointment was

administered; edema on extremities and systemic erythema improved and peripheral eosinophil count gradually decreased (500 to 3,000/μL), but the high levels of serum IgE persisted. The patient was discharged on February 11, 1998 with high levels of serum IgE (133,870 IU/mL).

MATERIALS AND METHODS

Antibodies and Reagents

FITC-conjugated mAbs against CD9 (mouse IgG1, Pharmingen, San Diego, USA), CD20 (mouse IgG1, Dakopatts A/S, Denmark), CD25 (mouse IgG1, Dakopatts A/S), CD54 (mouse IgG1, Immunotech SA, Marseille, France), CD69 (mouse IgG1, Becton Dickinson, San Jose, USA), VLA-4 (mouse IgG1, Immunotech SA) and phyco-erythrin (PE)-conjugated mAbs against HLA-DR (mouse IgG1, Becton Dickinson), CD4 (mouse IgG1, Becton Dickinson), isotype-matched FITC- or PE-conjugated mouse IgG1 mAbs (Dako patts A/S), purified anti-CD40 mAb (IgG1, Immunotech SA), recombinant human (rh) IL-4 (Genzyme Corp, Boston, USA) were purchased.

Cell Preparation

Peripheral blood mononuclear cells (PBMNCs) from the patient and normal subjects were isolated by Ficoll-Hypaque (Pharmacia, NJ, USA) density gradient centrifugation. Furthermore, erythrocyte rosette-positive (E+) and negative (E−) populations[12] were separated with 5% sheep erythrocytes. After depleting monocytes with silica (IBL, Fujioka, Japan) or adherence to the plastic surface, E− cells were further purified into B cells by positive selection with anti-CD19 mAb-coated immunomagnetic beads (Dynal, Oslo, Norway); anti-CD19 mAb was then removed by use of Detach a bead (Dynal). Ninety-seven percent of B cells were reactive with anti-CD20 mAbs. The B cells thus obtained had some tyrosine phosphorylations, whereas no proliferation and activation were found.

Cytokine and Chemokine Measurements

Peripheral blood (PB) was taken from the patient before steroid treatment. Peripheral blood was kept at room temperature for 30 minutes. Serum for cytokine measurement was obtained by centrifugation at 400 g for 10 minutes at 4°C. These samples were kept at −70°C until assay. Cytokine concentration was measured using an ELISA kit. The kits for tumor necrosis factor-α (TNF-α) (Medgenix Diagnostics, Fleurus, Belgium), interleukin-3 (IL-3) (Amersham International plc. Buckinghamshire, UK), interleukin-4 (IL-4) (Amersham International plc.) and interleukin-5 (IL-5) (R&D System, Minneapolis, USA), granulocyte/macrophage colony stimulating factor (GM-CSF) (Amersham International plc.), macrophage/monocyte chemo-tactic protein-3 (MCP)-3 (Toray Co, Tokyo, Japan) and regulated on activation, normal T cell expressed and secreted (RANTES) (Amersham International plc.) were employed. The minimum measurable amounts of TNF-α, IL-3, IL-4, IL-5 and GM-CSF are 6 pg/mL, 15 pg/mL, 15 pg/mL, 8 pg/mL, 5 pg/mL, 200 pg/mL and 50 pg/mL, respectively. Each cytokine level of normal control serum was below the minimum measurable sensitivity.

Surface Analysis

To analyze surface antigens of eosino-phils, we purified PB eosinophils using an improved immunomagnetic selection procedure.[13] Briefly, 20 mL heparinized venous peripheral blood was mixed with piperazine-N, N-bi (2-ethane sulfonic acid) (PIPES) buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, PH 7.4) at a 1:1 ratio. The diluted blood was overlayered on isotonic Percoll solution (1.082 g/mL) (Sigma) and centrifuged at 1,000 g for 30 minutes at 4°C. The mononuclear cell layer was then removed and erythrocytes in the sediment were lysed using two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with PIPES buffer with 1% inactivated fetal

calf serum (FCS) (Life Technologies, Inc, Gaithersburg, USA). The pellet of granulocytes was incubated with anti-CD16 mAb coated immunomagnetic particles (Miltenyi Biotec, Bergish-Gladbach, Germany; 50 μL for 5×10^7 cells) for 60 minutes at 4°C. Magnetically labeled neutrophils were then depleted by passing the granulocytes through a magnetic cell separation (MACS) column in the field of a permanent magnet (Miltenyi Biotec). This procedure resulted in more than 98% pure eosinophils with less than 2% contamination by neutrophils and without lymphocytes; viability was more than 98%. The purity and viability of eosinophils was established using Randolf staining and trypan blue dye exclusion, respectively. Ten or twenty microlitres of FITC-conjugated mAbs against CD9, CD25, CD54, CD69, VLA-4 and phycoerythrin (PE)-conjugated mAbs against HLA-DR, CD4 and isotype-matched FITC- or PE-conjugated mouse IgG1 mAbs were reacted with PB eosinophils ($5 \times 10^5/50$ μL) for 30 minutes at 4°C. Peripheral blood eosinophils were washed twice with PBS containing 1% inactivated FCS at 300 g for 10 minutes. The cells were resuspended in PBS containing 1% inactivated FCS. Gated area by forward light scattering and side light scattering confirmed the presence of eosinophils using anti-CD9 and anti-VLA-4 mAbs by flow cytometry (FACScan, Becton Dickinson). Surface analysis was then performed as reported previously.[13]

Immunoglobulin Assay by ELISA

Peripheral blood mononuclear cells were cultured with 50 ng/mL of IL-4 and 1 μg/mL of anti-CD40 mAb at a final cell density of 0.125 to 2×10^6/mL in a volume of 200 μL/well for 12 to 14 days at 37°C in a humidified atmosphere with 5% CO_2. Highly purified peripheral blood B cells (0.5 to 2×10^5/mL) were also cultured with or without his E+ cells (50% of purified B cells, 0.25 to 1×10^5/mL). The cultured supernatants were harvested, and the supernatants and the standard human IgE (Chemicon International

Inc, Temecula, CA, USA) were added to human IgE mAbs (CIA-E-7.12 and CIA-E-4.15, kindly provided by A. Saxon, Division of Clinical Immunology/Allergy, UCLA School of Medicine, Los Angeles, CA) and applied to 96 well flat ELISA plates (Nunc, Roskilde, Denmark). After overnight culture at 4°C, the supernatants were discarded and the wells were washed with 0.05% tween 20 in phosphate buffered saline (PBS); alkaline phosphatase-labeled goat anti-human IgE (Sigma Chemical Co, St. Louis, MO) was then added at a dilution of 1/5,000. After 2 hours incubation at room temperature, color detection was performed using 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer containing p-nitrophenyl phosphate (pNPP) (Sigma). Calibration was performed with PBS at standard zero levels. All experiments were performed in duplicate. The minimum measurable amount of IgE is 8 ng/mL.[14,15]

RESULTS

Cytokine and Chemokine Measurements

To clarify the mechanism of the eosinophilia and extremely high levels of serum IgE, we initially examined serum concentrations of several cytokines which affect eosinophilia or IgE synthesis. The level of TNF-α in serum was 21 pg/mL, while IL-3, IL-4, IL-5, and GM-CSF could not be detected. The levels of MCP-3 and RANTES were 0.8 ng/mL and 28.5 ng/mL, respectively. These levels were within the range of normal subjects (MCP-3: 1.1 ± 0.5 ng/mL; RANTES 27.8 ± 4.6 ng/mL).

Surface Analysis of Eosinophils

To confirm the presence of eosinophils antibodies against CD9 and VLA-4 were used for cytometric analysis. CD9 and VLA-4 were expressed on the cells in the gated area, showing these cells were eosinophils (Fig 1a). It was reported previously that CD4, CD25, HLA-DR, CD54 and CD69 do not express on eosinophils of normal subjects. As shown in Figures 1a and b, the profiles of CD25 (30%), CD69

(13%), CD4 (13%), and HLA-DR (35%), but not CD54 were significantly different from those of isotype matched control, demonstrating them to be newly expressed antigens on the eosinophils. CD25, CD69, CD4, CD54 and HLA-DR on a normal subject's eosinophils were not detected in parallel analysis.

Spontaneous IgE Production

Since the patient's serum contained extremely high levels of IgE, we thoroughly investigated IgE production. As shown in Figure 2, PBMNCs obtained from the patient produced high levels of IgE in a cell number dependent fashion without any stimulation. In contrast, PBMNCs obtained from normal subjects produced low levels of IgE with IL-4 plus anti-CD40 mAb stimulation (n = 10), whereas no IgE production (<8 ng/mL) was recognized in medium alone or IL-4 alone (Fig 2). Interestingly, the addition of IL-4 or IL-4 plus anti-CD40 mAb did not enhance IgE secretion compared with medium alone in the patient, indicating spontaneous production of IgE by his PBMNCs. We proceeded to examine IgE production by using highly purified B cells from the patient's peripheral blood. As shown in Figure 3, highly purified B cells produced high levels of IgE in a cell number dependent fashion; this production was not affected by the addition of IL-4 or IL-4 plus anti-CD40 mAb (data not shown). Furthermore, IgE production was not enhanced by the addition of T cells obtained from the patient into purified B cells. Purified B cells obtained from normal subjects showed low levels of IgE production with IL-4 plus CD40 signaling (purified B cells 1×10^4/well: 10 ± 2 ng/mL; 2×10^4/well: 16 ± 7 ng/mL; 4×10^4/well: 21 ± 9 ng/mL, n = 5), but no IgE production with medium alone (Fig 3) or IL-4 alone (data not shown). These data demonstrate that B cells from the patient spontaneously produced IgE.

DISCUSSION

Hyper-IgE syndrome is characterized by an immunodeficiency disease which

causes frequent staphylococcal abscesses, persistent eczematoid rashes, and extreme elevations of IgE in serum. Several observations such as erythema, positive acid fast staining, eosinophilia, high level of IgE in serum of the patient were similar to previous reports.[16,17] Recently, it was reported that hyper-IgE syndrome is an autosomal dominant multisystem disorder,[17] however the patient's family shows no features of the hyper-IgE syndrome.

Eosinophilia is observed in various diseases such as bronchial asthma, helminthic infection,[18] hyper-IgE syndrome[16,17] and in IL-2 administration.[19] Interleukin-3, IL-5, and GM-CSF are active in stimulating eosinophilopoiesis.[5] Interleukin-5 transgenic mice have developed massive, life-long eosinophilia,[20] suggesting IL-5 would be involved in a candidate mechanism for explaining hypereosinophilia; however, these cytokines were not detected in the patient's serum. The mechanisms of eosinophilia were not clear in this patient, suggesting other eosinophilic factor(s) were involved.

It is reported that numerous surface antigens are constitutively expressed on human eosinophils.[5] Hypodense eosinophils exhibit various morphologic changes such as cytoplasmic vacuolization, alterations in granules of major basic protein-containing cores or matrix.[18] These morphologic changes are well correlated with activated eosinophils induced by cytokines' (IL-3, IL-5 and GM-CSF) stimulation in vitro. There are several reports concerning newly expressed antigens in a variety of diseases. Expression of CD4 and CD25 on PB eosinophils was reported in hypereosinophilic syndrome (HES), helminth infection, and melanoma or renal cell carcinoma treated with IL-2.[19,21] CD69 expression on PB and bronchoalveolar lavage fluid (BALF) eosinophils was reported in HES and helminth infection.[22] These findings suggest that activated heterogenous eosinophils are present in various eosinophilic disorders. In this case, CD4, CD25, CD69 and HLA-DR, but not CD54, are expressed on eosinophils.
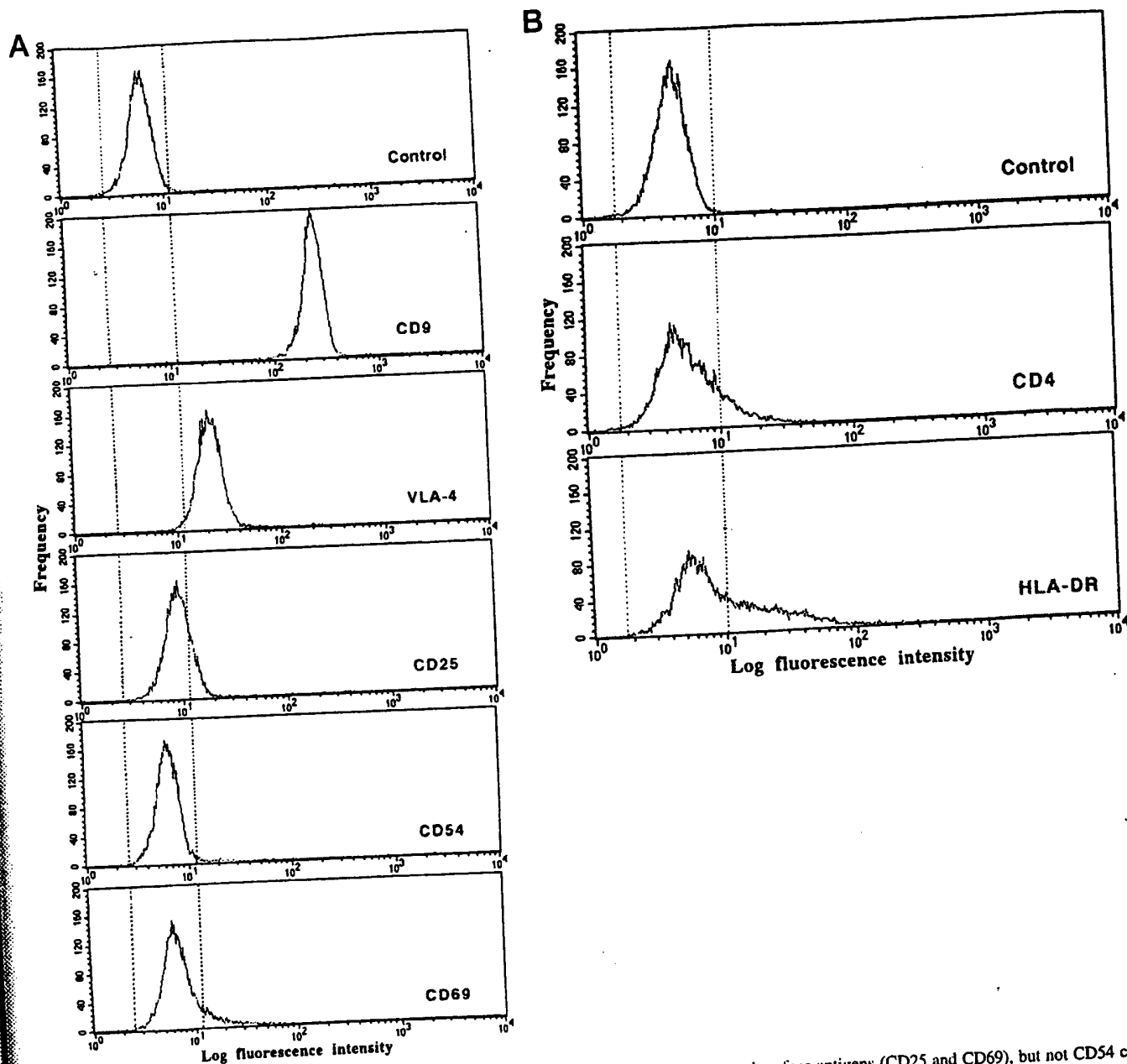
Figure 1. Surface analysis of peripheral blood eosinophils using flow cytometry. Newly expressed surface antigens (CD25 and CD69), but not CD54 could be detected (Fig 1a). Newly expressed surface antigens (CD4 and HLA-DR) could be detected (Fig 1b).

The reason is unclear, however the interaction of cytokines and chemokines may be involved in expression of eosinophil surface antigens.

IgE production of B cells is regulated by cytokines such as IL-4,[3,23,24] IL-13,[25,26] and direct contact between B and helper T cells,[2,24,27,28] in which CD40/CD154 interaction plays a key role in IgE synthesis.[3] Although it is known that IL-4 has a potent function in the production of IgE,[29,30] IL-4 alone induced germline ε transcripts, but not IgE production. Since our patient's B cells produced IgE spontaneously, we investigated germline ε transcripts using the patient's E-cells without stimuli; however, germline ε transcripts were not observed (data not shown). There is a possibility that, to our regret, we could not obtain enough B cells. EB virus-transformed B cell lines established from the patient also produced IgE spontaneously at first, but
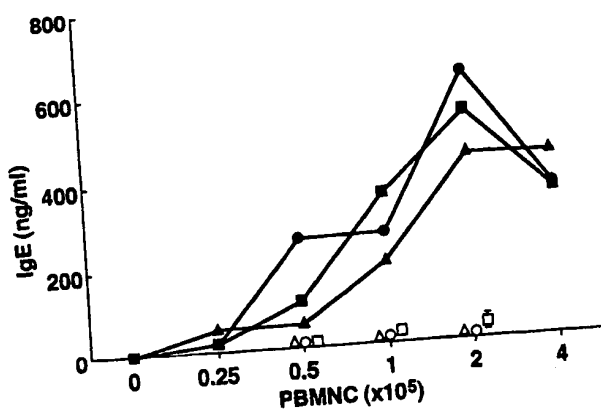
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Figure 2. IgE production by peripheral blood mononuclear cells (PBMNCs) of the patient and normal subjects (n = 10). Peripheral blood mononuclear cells were cultured with medium (▲: patient and △: normal subjects), IL-4 (●: patient and ○: normal subjects), or IL-4 plus anti-CD40 mAb (■: patient and □: normal subjects) at various concentrations in 96-well round-bottom plates for 12 to 14 days. IgE contents were measured using ELISA. Results are expressed as the mean ± SE of triplicates, and error bars are sometimes smaller than the plot symbol.
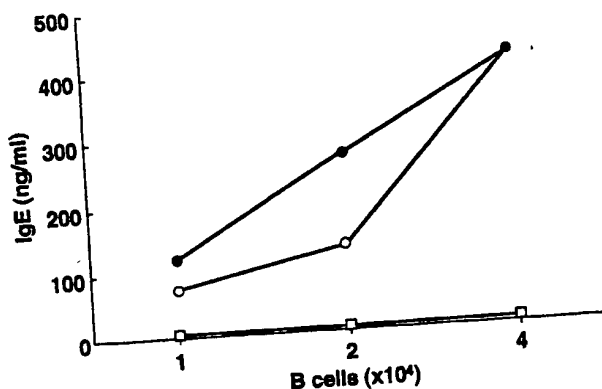

Figure 3. IgE production by purified B cells of the patient and normal subjects (n = 5). Highly purified B cells were cultured with medium alone (○: patient and □: normal subjects) and patient's purified B cells were also cultured with autologous patient's T cells (●) at various concentrations in 96-well round-bottom plates for 12 to 14 days. IgE contents were measured using ELISA. Results are expressed as the mean ± SE of triplicates.

sponsiveness of IL-4 in IgE production is due to variant IL-4 receptor.[32] We did not examine the variant IL-4 receptor in the patient. Grimbacher et al reported that IL-4 receptor variant allele was not significantly different from control subjects, suggesting IL-4 receptor gene is not linked to the hyper-IgE syndrome.[32]

In conclusion, we report a patient with remarkable eosinophilia and hyper-IgE. Although several cytokines were not detected in the serum, the eosinophils in peripheral blood were activated and the B cells spontaneously produce large amounts of IgE in vitro. Further analyses about the patient are needed to clarify mechanisms of eosinophil activation and IgE production, which will contribute the treatment of allergic diseases.

not EB virus-transformed B cell lines obtained from normal volunteers (n = 5), whereas the IgE production was gradually decreased and disappeared according to the culture (data not shown). Other immunologic studies of hyper-IgE syndrome have reported little responsiveness of exogenous IL-4 for IgE production.[31] Claassen et al reported neither PBMNCs nor B cells from the patients with hyper-IgE syndrome spontaneously produce IgE in vitro, little responsiveness of exogenous IL-4 for IgE production and re-

quirement of high concentration of anti-CD40 for IgE production.[31] Our data presented here are clearly different from the report by Claassen et al in the point of spontaneous IgE production of PBMNCs and B cells and no response of anti-CD40 for IgE production, however, it is similar to the data to be little responsiveness of exogenous IL-4 for IgE production. It is considered that once B cells become committed to IgE synthesis, IL-4 appears to have little role in the regulation of IgE production. Another possibility in low re-

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Anti-inflammatory activity of nerve growth factor in experimental autoimmune encephalomyelitis: inhibition of monocyte transendothelial migration

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In order to analyze a putative immunomodulatory effect of NGF in experimental autoimmune encephalomyelitis (EAE) of the Lewis rat, we transduced myelin basic protein (MBP)-specific CD4$^+$ T cells with a recombinant retrovirus encoding NGF. These $T_{MBP}NGF$ cells secreted high levels of NGF, along with an unaltered Th1-like cytokine pattern. Transfer studies showed that $T_{MBP}NGF$ cells were unable to mediate clinical EAE, when transferred alone, and, more important, they efficiently suppressed induction of clinical EAE by non-transduced MBP-specific T cells (T_{MBP} cells). In contrast, NGF transduced ovalbumin-specific T cells, which secreted high NGF levels, did not affect EAE induction. Suppression of clinical EAE by $T_{MBP}NGF$ cells was associated with a general reduction of inflammatory CNS infiltrates, with a most pronounced decrease of the monocyte/macrophage component. Using a culture model of the endothelial blood-brain barrier (BBB), we found that NGF directly acts on blood-derived monocytes via the p75 NGF receptor, thus interfering with monocyte migration through the activated BBB endothelium. Our data establish NGF as an anti-inflammatory mediator interfering with T cell mediated autoimmune disease in the CNS. They further point to monocyte migration through blood vascular endothelium as one possible mechanism of NGF action.

1 Introduction

The neurotrophins, a family of related polypeptides which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), exhibit a remarkable range of biological activities. Apart from controlling development, differentiation, and functional plasticity of neurons [1, 2], neurotrophins are also involved in the regulation of inflammatory and immune responses [3].

Recently, we demonstrated that NGF released by neurons suppresses the inducibility of MHC class II in micro-

The first two authors contributed equally to this work.

Abbreviations: BBB: Blood brain barrier **CNS:** Central nervous system **(t)EAE:** (transfer) Experimental autoimmune encephalomyelitis **FACS:** Fluorescence activated cell sorter **MBP:** Myelin basic protein **MS:** Multiple sclerosis **NGF:** Nerve growth factor

glial cells, and thus may interfere with immune responsiveness in the CNS [4]. In addition, there is increasing evidence that the factor can influence immune responses in the periphery, modulating the proliferation and differentiation of B [5–7], and possibly T cells [8, 9]. NGF is also reported to stimulate memory B cells and Th2 immune responses [5, 10, 11]. Recent studies further demonstrated that NGF could indirectly modulate inflammation by regulating pain reactivity [12, 13].

While the role of NGF in inflammatory responses is still incompletely understood, it appears that it functions as an anti-inflammatory agent in T cell-mediated disease. In a hind-paw edema test, NGF was shown to decrease inflammatory edema formation [14]. Then, NGF released from genetically modified neuritogenic T lymphocytes drastically reduced inflammatory cell infiltration in experimental autoimmune neuritis (EAN) of the Lewis rat [15]. Further, NGF administrated intracerebroventricularly into marmosets delayed onset of EAE and interfered with the formation of CNS lesions [16].

We now report that NGF released from genetically engineered T cells profoundly mitigates T cell-mediated autoimmune inflammation in the CNS. *In vivo*, transgene-derived NGF reduces inflammatory CNS infiltration, especially of monocytes and macrophages. *In vitro* results indicate that NGF acts on brain microvascular endothelial cells blocking transmigration of inflammatory cells.

2 Results

2.1 NGF production by autoreactive MBP-specific T cells

ELISA screening of activated antigen-specific CD4+, TCR-α/β+ T cell lines (TCL) isolated from primed Lewis rats following established protocols [17, 18] revealed that wild-type TCL secrete low, but consistent amounts of NGF ($\leq$20 pg/ml) in addition to typical Th1-like cytokines (Fig. 1).

We over-expressed NGF in T cell lines by infecting antigen primed T cells with recombinant retrovirus encoding the NGF gene. As described previously, the transduction of TCL did not affect cytokine production (Fig. 1), antigen reactivity and TCR usage [19]. However, the retrovirally transduced TCL released NGF-levels 10–15-fold higher than the wild-type TCL (Fig. 1). In addition, using confocal laser scanning microscopy we were able to visualize enhanced NGF levels in the cytoplasma of the modified cells (Fig. 2).

2.2 Suppression of T cell-mediated EAE by co-transfer of NGF-transduced CNS antigen-specific T cells

We examined the possible immunosuppressive effect of NGF-engineered, MBP-specific T cells (T_{MBP}NGF cells) using a classical T cell transfer model of EAE (tEAE). As expected, i.p. injection of wild-type MBP T cells (T_{MBP}cells) mediated the clinical hallmarks of transfer EAE (tEAE), characterized by ascendent paresis, urinary incontinence and weight loss (Fig. 3). In contrast, T_{MBP}NGF cells induced no or very mild clinical neurological disease and weight loss. Moreover, co-injection of T_{MBP}NGF cells suppressed the clinical symptoms of EAE elicited by wild-type T_{MBP}cells (Fig. 3). Specifically, rats injected with 2×10^6 wild-type T cells alone resulted in the clinical score 3 (complete hind limb paralysis), while animals co-injected with 2×10^6 wild-type MBP T cells plus 2×10^6 T_{MBP}NGF cells developed no or minor clinical symptoms, rarely reaching score 1 (Fig. 3A). Likewise, rats injected with wild-type plus NGF engineered T cells lost little, if any, weight. This protective effect of T_{MBP}NGF was maintained throughout the clinical disease course and into the recovery phase of tEAE (Fig. 3A).

Importantly, only MBP-specific, NGF-transduced T cell lines had a suppressive potential in tEAE. Ovalbumin-specific, NGF-engineered T cells (T_{OVA}NGF cells) did not influence the clinical course of tEAE evoked by wild-type MBP-reactive T cells. T_{OVA}NGF cells interfered at no stage with the development of tEAE (Fig. 3B).

2.3 NGF-transduced T cells reduce numbers of monocytes/macrophages and MHC class II-positive cells in EAE lesions

The protective effect of T_{MBP}NGF cells was further examined by histology. Five days after transfer of wild-type
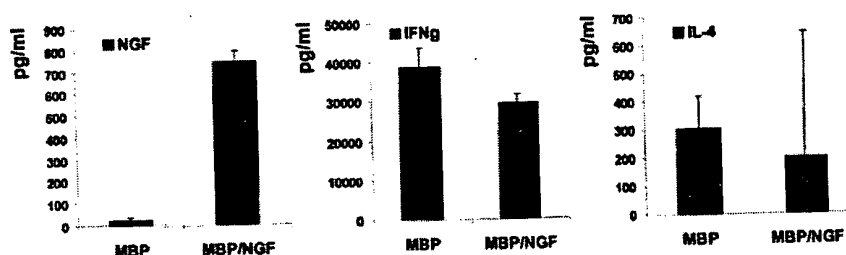

Fig. 1. Left panel: NGF production of MBP-specific T cell blasts before (MBP) and after retroviral transduction with a NGF-encoding virus (MBP/NGF). Middle and right panels: IFN-γ (middle panel) and IL-4 (right panel) expression of T_{MBP} blasts and T_{MBP}NGF blasts. The NGF and cytokine levels were determined from 24 h supernatants of T cell lines (1×10^6/ml). Data are presented as mean ± SD.
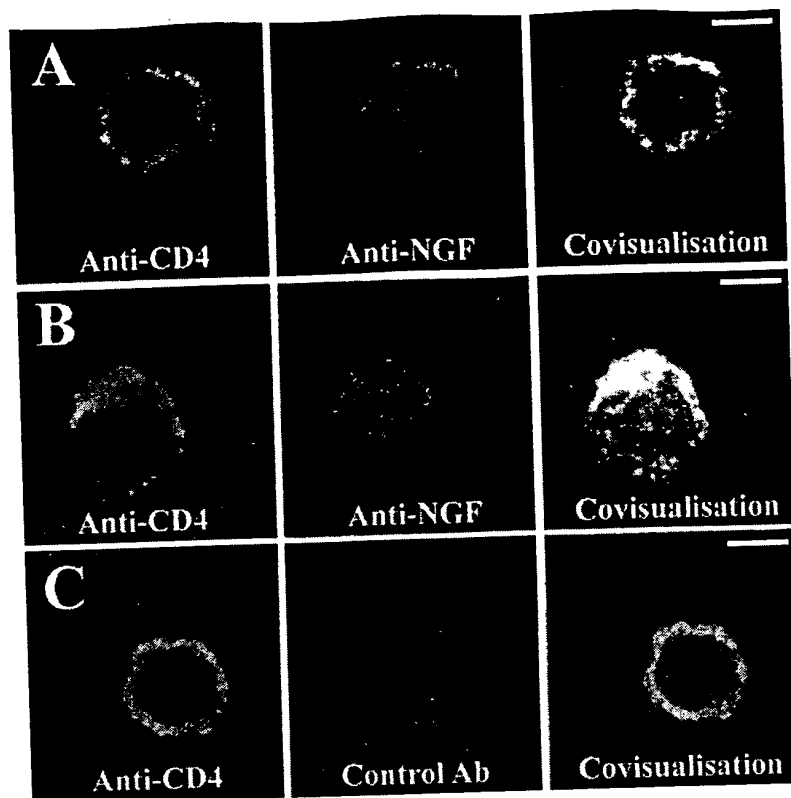
Fig. 2. Confocal immunohistochemistry of T_{MBP}cells with antibodies directed against NGF (red). T cells were double labeled with antibodies directed against CD4 (green). Non-transduced MBP-specific T cells (A and C). NGF-transduced MBP-specific T cells (B). Background immunolabeling was determined with primary mouse monoclonal isotype control antibodies (C). Scale bar: 5 μm.

T_{MBP}cells, the recipients showed typical histopathology of tEAE. Transfer of engineered T_{MBP}NGF cells alone produced no or very mild CNS inflammation. Combined transfer of wild-type plus T_{MBP}NGF cells substantially diminished the inflammatory response in the CNS. The number of MHC class II-positive cells in the CNS of all animals, which had received T_{MBP}NGF cells was reduced from 490 ($\pm$ 122 SD) in rats treated with wild-type T_{MBP} cells to 52 ($\pm$ 43 SD) in rats co-injected with T_{MBP} cells and T_{MBP}NGF cells (Table 1). Moreover, quantification of spinal cord sections for ED1$^+$ monocytes/macrophages and W3/13$^+$ T cells revealed that suppression of CNS inflammation was strongly biased in favor of the monocyte/macrophage population increasing the T cell/macrophage ratio from 0.8 (group of 1.5×10^6 T_{MBP} cells) to 2.2 (group of 1.5×10^6 T_{MBP} cells co-injected with 1.5×10^6 T_{MBP}NGF cells) in the residue infiltrates (Table 1).

2.4 Expression of NGF receptors on monocytes

To study the therapeutic NGF effect on CNS infiltrating, blood-derived monocytes/macrophages, we first determined the expression of both high (trkA) and low affinity (p75) NGF receptors by RT-PCR (Fig. 4A). PCR amplification of monocyte cDNA yielded a single band of the size (353 bp) expected for p75^{NTR} mRNA. The identity of the PCR product was confirmed by direct sequencing. In contrast, no trkA-specific mRNA was amplified in our monocyte preparations after 35 cycles PCR, indicating no or very low expression as compared to dorsal root ganglion (DRG) neurons as a positive control. Expression of p75^{NTR} in monocytes was confirmed on protein level by immunofluorescence staining and flow cytometry (Fig. 4B). The purified monocytes identified with the mAb OX42 were intensely stained with a mouse mAb directed against rat p75^{NTR}.
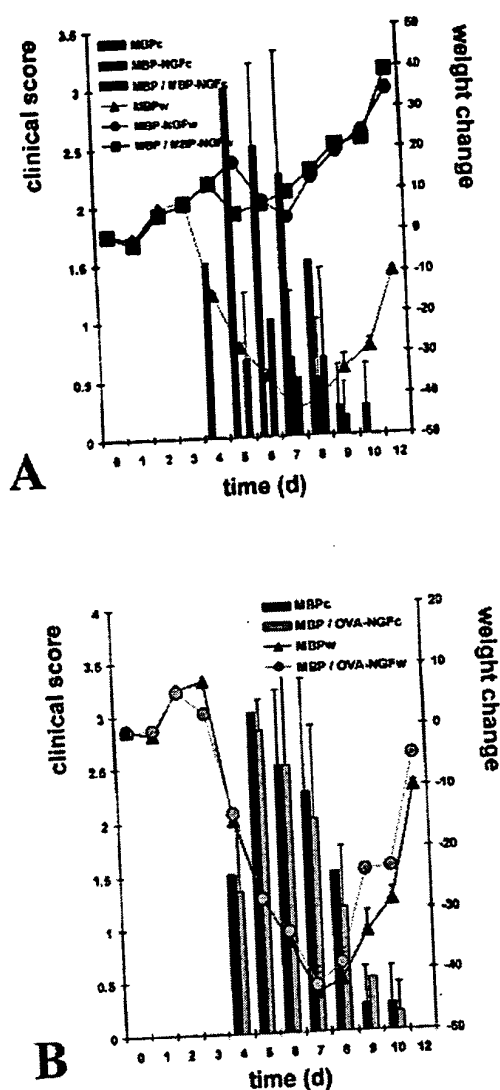
A

B

2.5 NGF inhibition of monocyte migration through TNF-activated brain endothelial cell monolayer

Functional effects of NGF on monocyte migration was analyzed in an *in vitro* model of the endothelial blood-brain barrier (BBB) (Fig. 5). Brain microvascular endothelium from adult Lewis rats was cultured in a two-chamber system. The purity of the endothelial cell cultures was over 95% by immunofluorescence staining for factor VIII-like antigen [20]. The endothelial cells used for transmigration assay after the first passage showed the typically elongated spindle like appearance often forming whorls (Fig. 5A). In these cultures, transendothelial
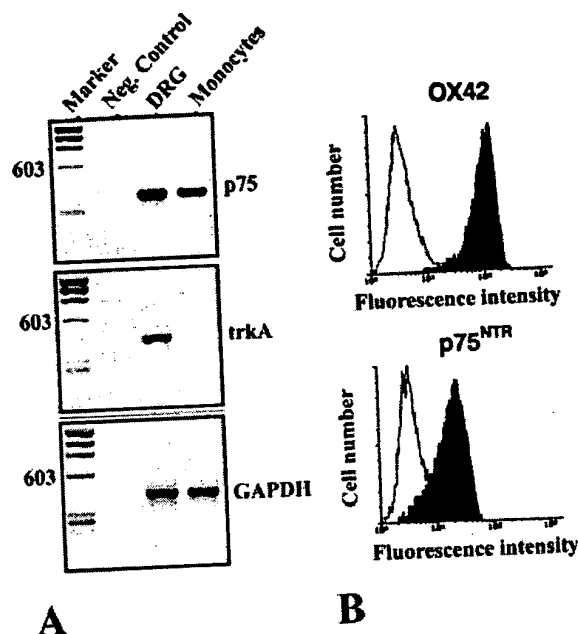

A

B

Fig. 3. T_{MBP}NGF cells, but not T_{OVA}NGF cells suppress disease induced by wild-type T_{MBP} cells. Weight course in relation to day 0 (curves, second y-axis) and clinical score (bars, first y-axis) were analyzed daily following intraperitoneal transfer of T cells. Each value represents the mean and standard deviation of at least three animals. Development of EAE symptoms following transfer of: (A) 2×10^6 T_{MBP} cells (blue triangles and bars), 2×10^6 T_{MBP}NGF cells (green circles and bars), and 2×10^6 T_{MBP} cells co-injected with 2×10^6 T_{MBP}NGF cells (red squares and bars). The ameliorating effect of T_{MBP}NGF cells can be observed throughout the entire clinical course of EAE. (B) 2×10^6 T_{MBP} cells (blue triangles and bars) and 2×10^6 T_{MBP} cells co-injected with 2×10^6 T_{OVA}NGF cells (orange circles and bars).

Fig. 4. (A) NGF receptor expression in rat blood monocytes. Total RNA isolated from highly purified monocytes was reverse-transcribed and PCR-amplified (35 cycles) using primers specific for $p75^{NTR}$ and trkA. RNA extracted from rat dorsal root ganglia cultures (DRG) served as positive control, PCR-reactions lacking cDNA served as negative control (Neg. Control). PCR amplification (30 cycles) of GAPDH served as control for RNA input and amplification. RT-PCR analysis demonstrated gene transcript for the p75 ^{NTR} in rat monocytes. In contrast, no trkA mRNA was detected in the monocyte preparation. DNA molecular size marker: ϕX174/Hae III digest. (B) $p75^{NTR}$ protein expression by rat peripheral blood monocytes. Isolated monocytes were incubated with mouse anti-rat $p75^{NTR}$ mAb (closed curve) or control mouse IgG (open curve) followed by fluorescein-conjugated second antibody. These purified cells were also stained with OX42 mAb as a marker for monocytes (closed curve). Purified cells were positive for OX42 and they also expressed $p75^{NTR}$.

Table 1. Quantification of monocyte/macrophage and T cell infiltration and MHC class II expression in the spinal cord

	Inf. Index[a]	W3/13+ T cells (std)	ED1+ Monocytes/ macrophages (std)	OX-6+ cells	Ratio W3/13 / ED1
T-MBP (1.5 × 10⁶)	3.8 (1.5)	349 (4)	449 (47)	490 (122)	0.8
T-MBP (3 × 10⁶)	4.9 (1.8)	556 (265)	360 (58)	450 (195)	1.5
T$_{NGF}$-MBP (1.5 × 10⁶)	0.01 (0.01)	0.7 (0.2)	0.3 (0.1)	15 (10)	2.2
T$_{NGF}$-MBP (3 × 10⁶)	0.1 (0.2)	24 (41.9)	8.9 (14.8)	12 (6)	2.8
T-MBP (1.5 × 10⁶) + T$_{NGF}$-MBP (1.5 × 10⁶)	0.3 (0.2)	51 (61)	23.7 (25.2)	52 (43)	2.2

a) Inflammatory index (Inf. Index): number of inflammatory infiltrates/spinal cord cross section. Number of T cells, ED1 and OX-6+ cells are given per mm². At least three animals/group and 10 sections/animal covering all spinal cord levels were analyzed. The values represent means and standard deviations ( ) of at least 30 sections.

electrical resistance, a marker for confluency and the formation of tight junctions, usually reached 30 Ω/cm².

Activation of brain vascular endothelial cells with 10 ng/ml TNF-α for 24 h induced VCAM-1 and ICAM-1 expression and significantly promoted the transmigration of monocytes (Fig. 5C).

The effect of NGF on monocyte migration through TNF-α-activated brain endothelial cell monolayer was examined by pre-treating monocytes and endothelial cells separately. Pretreatment of monocytes with 100 ng/ml NGF resulted in sustained inhibition of their migratory capacity (Fig. 5C). The transmigration through activated endothelial cell monolayers was reduced from 10.4±0.7% (mean ± SD) in untreated monocytes to 4.4±0.8% in monocytes pretreated with 100 ng/ml NGF (Fig. 5C). NGF reduced monocyte migration through TNF-α-activated but not through resting endothelial cell monolayers (Fig. 5C). NGF affected monocyte migration even at a concentration of 1 ng/ml (Fig. 6A). This inhibitory effect on monocyte function was reversed by neutralizing the neurotrophin with NGF-specific antibody pretreatment (Fig. 6B). NGF-pretreatment of brain endothelium (resting or TNF-α-activated) did not modulate monocyte transmigration (data not shown).

To confirm that the NGF effect on monocyte transendothelial migration was mediated via p75^{NTR}, we used a blocking antisera binding to an extracellular p75^{NTR} epitope [21]. Pretreatment of rat monocytes with this anti-p75^{NTR} antiserum completely neutralized the inhibitory effect of NGF on monocyte migration through TNF-α-activated endothelial cell monolayers (Fig. 6C).

3 Discussion

Immune reactivity in the CNS milieu is generally lower than in other tissues. One factor that contributes to this *immune privilege* is the lack of locally expressed MHC determinants, as a prerequisite for antigen presentation. Recently, we identified NGF mediating a suppressive effect on the IFN-γ-dependent induction of MHC expression in the CNS [4]. The results presented here confirm the immunosuppressive potential of NGF *in vivo*, and reveal a second mechanism of immune modulation. We show that NGF can prevent migrant monocyte or macrophages from crossing the BBB and thus inhibits the formation of pathogenic inflammatory infiltrates.

Our *in vivo* experiments were based on a retroviral gene transfer strategy involving co-culture of myelin primed T cells with autoantigen, virus-producing packaging cell lines and a selecting antibiotic. The surviving T cells were efficiently transduced (> 99%) [19] and the transgene expression was activation dependent. While unmodified CD4+ T cells produce significant amounts of NGF [8] and other neurotrophins [22, 23], T$_{MBP}$NGF cells produced 10–15-fold higher levels of NGF than the wild type, and fourfold higher levels than resting transduced T cells (not shown). It is important to note that NGF transduction neither interfered with antigen reactivity and specificity, nor did it change the CD4+, $\alpha\beta$T cell receptor phenotype and Th1-like cytokine profile. NGF transduced and non-transduced T cells both expressed high amounts of IFN-γ and low amounts for IL-4 (Fig. 1). Transduction of MBP-reactive T cells with a different neurotrophic agent, GDNF, did not change the EAE inducibility and severity of the disease (unpublished observations). The data confirm our previous observations with green fluorescent protein-transduced T cells, showing that the transduc-
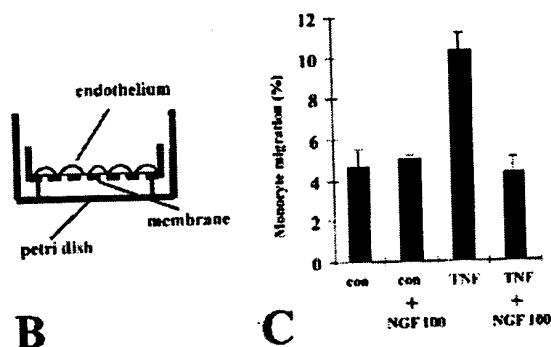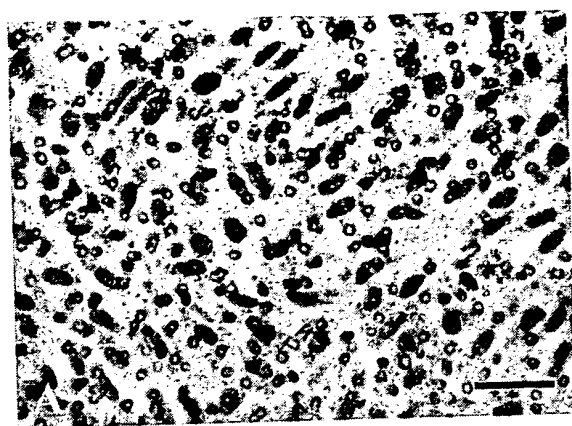
Fig. 5. (A) The brain endothelial cells on the microporous membrane show typical elongated spindle like appearance that made a whorl formation. Giemsa staining. Scale bar: 50 µm. (B) Two chamber transendothelium migration system. (C) Characterization of rat monocyte migration through resting versus TNF-α-activated brain endothelial cell monolayer. The brain endothelial cells were incubated in the medium alone (con) or in the presence of 10 ng/ml of TNF-α (TNF) for 24 h. Monocytes (4×10⁵) were put into the upper chamber and migration of these cells were assessed after 12 h. Pretreatment of the brain endothelial cell monolayer with TNF-α resulted in a significant enhancement in the migration of monocytes. NGF attenuates the migration of monocytes through TNF-α-activated brain endothelial cell monolayer. Monocytes were incubated in the medium alone or in the presence of 100 ng/ml of NGF for 4 h and put onto the upper chamber (NGF 100). Pretreatment of monocytes with NGF down-modulated their migration through TNF-α-activated brain endothelial cell monolayer. Data are expressed as the mean ± SD of the percent migration calculated from triplicate wells.

Fig. 6. (A) Monocytes were pretreated for 4 h with the indicated concentrations of NGF. The reduction of NGF-treated monocyte migration was apparent at NGF concentration of 1 ng/ml or higher. Data are expressed as the mean ± SD of the percent migration calculated from triplicate wells. (B) Neutralization of NGF with specific antibodies (Anti-NGF) reversed the inhibitory effect of NGF (100 ng/ml) on monocyte transendothelium migration. (nt): non-treated monocytes. Data are expressed as the mean ± SD of the percent migration calculated from triplicate wells. (C) NGF modulates monocyte transendothelial migration via p75 NTR. Purified monocytes were treated with anti- p75 NTR antiserum (Anti-p75) for 1 h and subsequently incubated with 100 ng/ml of NGF for 4 h. The attenuation of the monocyte migration through TNF-α-activated brain endothelial cell monolayer by NGF was blocked by pretreatment of those monocytes with anti-p75NTR antiserum. (nt): non-treated monocytes. Data are expressed as the mean ± SD of the percent migration calculated from triplicate wells.

tion procedure does not interfere with the encephalitogenic potential of T cells [19].

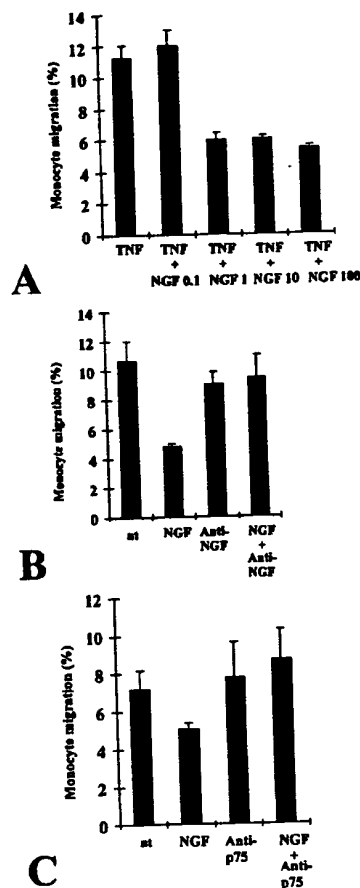NGF transduction of MBP-specific T cells profoundly curtailed their encephalitogenic potential, and moreover, NGF overproduction suppressed the pathogenic activity of co-transferred MBP-specific wild-type T cells. Using NGF-engineered P2 protein-specific T cells comparable observations were made in an experimental autoimmune neuritis model [15].

Our present results suggest locally deposited NGF rather than a systemic effect being responsible for the anti-inflammatory capabilities of this neurotrophin: First, comparing two transduced T cell lines secreting comparable amounts of transgenic NGF we found that only MBP-specific, but not ovalbumin-specific transduced T lymphocytes mediated suppression of EAE. Secondly, soluble NGF titers in the blood were not significantly elevated in rats after transfer of T_{MBP}NGF cells compared to wild-type T cells (unpublished observations). Thirdly, NGF-mediated immune deviation of encephalitogenic T cells is not a probable mechanism, since transduction did not demonstrably alter the Th1-like phenotype of MBP-specific T cells. Recently, evidence has been presented that stereotactic application of NGF to myelin oligodendrocyte glycoprotein (MOG) -immunized marmosets delays clinical disease and reduces EAE pathology [16]. Immunohistochemical analysis in this study showing reduced IFN-γ and increased IL-10 staining in EAE lesions was interpreted as NGF-induced cytokine shift in T cell response. In contrast, our study is based on NGF-producing TH1 T cells as biological shuttle and clearly favors a different modulatory NGF mechanism.

T_{MBP}NGF cells drastically reduced the number of inflammatory cells crossing the endothelial BBB with a particular decrease of activated macrophages. Interestingly, the number of activated macrophages is associated with the clinical severity of T cell mediated EAE [24], and depletion of macrophages profoundly reduces inducibility of EAE [25, 26].

T_{MBP}NGF cells could interfere in several ways with infiltration of activated monocytes/macrophages. Complete antigen activation of encephalitogenic T cells in situ could be prevented by the NGF-dependent suppression of MHC induction [4]. As a consequence, T cell activation induced chemokines or cytokines mediating monocyte/macrophage recruitment could be insufficiently produced or absent. Indeed, we found strongly reduced numbers of MHC class II-expressing cells in spinal cords of animals which had received T_{MBP}NGF cells (Table 1). The low MHC molecule expression could, however, well be explained by the strong decrease in monocyte/macrophage infiltration. Alternatively (or in addition) NGF could act either on BBB endothelial cells or directly on the recruitable inflammatory cells. Our *in vitro* data support this later assumption.

The effect of NGF on BBB migration was analyzed using an *in vitro* migration assay consisting of a monolayer of brain endothelium separating two chambers [27]. The endothelial cells, which were derived from highly purified, primary brain microvascular cultures, showed all morphological and functional features (*e.g.*, intercellular electric resistance) characteristic of specialized BBB-derived endothelium [28]. The endothelia were activated with TNF-α to simulate the state of activation seen in inflammatory brain lesions [29, 30].

We found that NGF acted on the migrant monocytes, but not on the endothelial cells. The anti-migratory effect of NGF on monocytes was limited to their passage through TNF-α-activated brain endothelial cell monolayer, and was not seen with resting endothelia. In contrast, no essential effect of NGF on T cell transmigration through resting or TNF-α-activated endothelium was observed (data not shown).

NGF acts on cellular targets by binding to two distinct sets of specific membrane receptors, the high affinity trkA and the low-affinity p75 neurotrophin receptor. The p75 receptor, a member of the TNF receptor family [31], is expressed in many cell types of the CNS and PNS, as well as in many non-neural tissues [32]. Neurotrophin receptor expression in monocytes is still controversial. There are reports describing either expression of trkA in macrophages/monocytes [33], or p75 [34]. In human monocytes an activation-dependent fluctuation between p75 and trkA has been reported [35, 36].

Examining highly purified rat blood monocytes by RT-PCR, we found only p75^{NTR} mRNA, but no detectable levels of trkA mRNA. The expression of p75^{NTR} protein was confirmed by cytofluorometry analysis of purified monocytes. Blocking tyrosine kinase activity of the trkA receptor with K252a did not interfere with the inhibitory activity of NGF on transendothelium migration of monocytes. Furthermore, the use of a blocking anti-p75^{NTR} antiserum finally corroborated that direct activation of p75^{NTR} was responsible for reducing monocyte transmigration.

In conclusion, our data demonstrate a new immunoregulatory function of NGF, down-regulating autoimmune T cell responses by reducing recruitment of accessory macrophages into the inflammatory lesion. Based on these results, cell therapy with NGF-transduced T cells might provide a valid therapeutic approach for MS.

4 Materials and methods

4.1 Reagents

NGF was purchased from Sigma (NGF-7S, Munich, Germany). Human recombinant TNF-α was a product of Biozol (Eching, Germany). mAb OX42 to rat monocyte/macrophage antigen (Mac-1 or CR3 complement receptor) was purchased from Dianova (Hamburg, Germany). mAb

to rat low affinity NGF-receptor (clone 192) was purchased from Roche Diagnostics (Mannheim, Germany). Isotype control antibodies (IgG1 and IgG2a) for flow cytometry were obtained from Becton Dickinson (Heidelberg, Germany). Antiserum directed against the p75 neurotrophin receptor (9651), was generously supplied by M. V. Chao, Cornell University, NY. Mouse monoclonal anti-CD4 and T cell receptor antibodies were obtained from PharMingen (Hamburg, Germany).

4.2 Production of T cell lines

MBP- and OVA-specific T lymphocytes were generated from Lewis rats after subcutaneous immunization with 150 µg guinea pig MBP or chicken egg ovalbumin (Sigma, Germany) emulsified in complete Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* (Statens Seruminstitut, Copenhagen, Denmark). Myelin basic protein (MBP) was purified from guinea pig brain using the protocol of Eylar and colleagues [37]. Inbred Lewis rats 6–8 weeks of age were obtained from the animal breeding facility of the Max-Planck-Institute of Biochemistry (Martinsried, Germany) and were cared for in accordance with all institutional guidelines. Ten days after immunization, cells were isolated from the draining popliteal and inguinal lymph nodes and cultured following a modified limiting dilution technique [18]. In brief, 2×10^5 cells/well were cultured in the presence of 10 µg/ml antigen in round-bottom 96-well plates (Nunc, Wiesbaden, Germany) for 3 days and propagated for another 6–8 days in IL-2 conditioned medium. For restimulation the T cells were co-cultured with 1×10^6 irradiated (50 Gy) syngeneic thymocytes/well as antigen presenting cells (APC). Further amplification of the T cells was performed as described (Ben-Nun et al. [17]).

4.3 Retroviral transduction of CD4+ T cells

The retroviral vector encoding the NGF gene and the packaging line producing replication-deficient NGF viruses were a kind gift from R. Kramer (Max-Planck-Institute for Neurobiology, Martinsried-Planegg). The NGF cDNA was cloned into the retroviral vector pLXSN [38]. As packaging cells the ecotropic packaging line GP+E 86 [39] was used. Transduction of lymphocytes was performed as described elsewhere [19]. In brief, GP+E 86 packaging cells producing replication deficient retrovirus (approx. 1×10^6CFU/ml) were seeded in 96-well plates (10^6/plate). Lymphocytes harvested from immunized animals were added to the wells (1×10^5/well). After 3 days of antigen stimulation, the T lymphocyte blasts were expanded in IL-2 containing growth medium. Selection with G418 (0.4 mg/ml) was started after 3 more

days in culture in the presence of IL-2 conditioned medium and maintained during subsequent restimulations. Neurotrophin and cytokine expression of the transduced TCL was controlled by NGF-ELISA (Roche Molecular Biochemicals, Mannheim, Germany), IFN-γ-ELISA and IL-4-ELISA (Biosource, Camarillo, USA).

4.4 Immunohistochemistry for NGF

Cytospin preparations of freshly activated MBP-specific T cell lines were fixed in 4% paraformaldehyde. After blocking unspecific binding sites with 2% goat serum and 2% BSA in PBS, cells were incubated with mouse mAb directed against NGF (1 µg/ml, Roche, Mannheim, Germany). To determine background labeling, cells were incubated with mouse isotype control antibodies (1 µg/ml, Becton Dickinson). Cells were then incubated with fluorochrome Cy3-conjugated polyclonal goat antibodies directed against mouse immunoglobulin (10 µg/ml, Dianova, Hamburg). Cells were sequentially double labeled with fluorochrome FITC-conjugated antibodies directed against rat CD4 (5 µg/ml, PharMingen). Images were scanned with confocal laser scanning microscopy (Leica) equipped with a 63 × oil objective.

4.5 EAE induction and histological analysis

Passive transfer EAE in animals was induced by intraperitoneal injection of encephalitogenic T lymphocytes. Weight and clinical score of the animals were observed and determined once every day. Evaluation of clinical disease after EAE induction was graded in 5 scores: 0.5: loss of tail tonus, 1: tail paralysis, 2: gait disturbance, 3: hind limb paralysis, 4: tetraparesis, 5: death.

For histological analysis of CNS tissue, animals were perfused with PBS/4% paraformaldehyde (PFA) under deep anesthesia. Isolated brain and spinal cord was then post-fixed in 4% PFA at 4°C overnight followed by embedding in paraffin. Sections (3 µm) were mounted on gelatinized slides, stained with hematoxylin/eosin or subjected to immunohistochemistry. W3/13 (recognizing T cells) and ED1 (recognizing monocytes/macrophages) were obtained from Dako (Glostrup, Denmark), OX6 (recognizing MHC class II) was obtained from Serotek (Oxford, GB). The antibodies were diluted in PBS containing 10% fetal calf serum (FCS) in the following dilution: ED-1 (1:500), W3/13 (1:500), OX6 (1:150). Bound primary antibodies were visualized with a biotin – avidin technique as described before [40].

4.6 Quantitative evaluation

The global inflammatory response in the spinal cord was determined in hematoxylin/eosin stained sections by counting the number of perivascular inflammatory infiltrates in an average of ten complete spinal cord cross sections from all levels of the cord. Values in Table 1 represent the average numbers of infiltrates by spinal cord cross sections. Immunostained T cells (W3/13), macrophages (ED1), and MHC class II (OX6) expressing cells were counted on three randomly selected complete spinal cord cross sections from the lower thoracic level. The section area was determined by overlaying the respective sections with a morphometrical grid. The values for the density of W3/13, ED1, and OX6 positive cells are given in cells/mm².

4.7 Monocyte purification

Peripheral blood monocytes were isolated by EDTA-reversible attachment to autologous serum-coated plastic surface [41]. Heparinized blood was taken by cardiac puncture from adult Lewis strain. Peripheral blood mononuclear cells were isolated by density gradient fractionation using Ficoll-Metrizoate solution (23 parts of 9% Ficoll 400, Pharmacia, Uppsala, Sweden, to 10 parts of 32.8% Sodium Metrizoate, Nycomed, Oslo, Norway). The interface cells were harvested, washed twice at $100 \times g$ and 4°C for removing platelets and resuspended in DMEM medium (Gibco BRL, Berlin, Germany) supplemented with 10% fetal calf serum (PanSystems, Nürnberg, Germany), 1% penicillin-streptomycin, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate and 1% L-asparagine (all from Gibco BRL). The cells were then allowed to adhere to rat serum-coated plastic dishes. Following incubation for 2 h, the dishes were rinsed with three changes of HEPES-buffered DMEM (E.H. medium). Adherent cells were removed by cold phosphate buffered saline (PBS) containing EDTA. May-Grünwald-Giemsa staining of the cells showed the complete absence of neutrophils with predominance of monocytes. The purity of the isolated monocytes was routinely more than 90%, as indicated by immunofluorescence flow cytometry using mAb OX42. Further enrichment of monocytes was achieved in some experiments by repeated washing of the dishes. Monocyte preparations then had about 99% cell viability as determined by trypan blue exclusion.

4.8 Reverse transcription combined with the polymerase chain reaction (RT-PCR) analysis

Highly purified monocytes (purity 99% as assessed by Ox42 surface marker analysis) were prepared for RT-PCR analysis. Total cellular RNA was isolated with RNA-zolB (Wak-chemie, Bad Homburg, Germany). Total RNA (1 µg) of each sample was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (100 units, Gibco BRL), dithiothreitol (10 mM, Gibco BRL), hexamer random primer (1 µl, Roche, Mannheim) and the four deoxyribonucleotides triphosphate (dNTPs, 0.5 mM, Pharmacia) in a total volume of 20 µl. The cDNA were amplified by PCR using specific primers for p75^{NTR} and trkA. PCR amplification for constitutively expressed mRNA encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the control for RNA input and amplification. PCR-amplification was done in a final volume of 50 µl containing 1 µl of transcribed cDNA probe, the four dNTPs (0.2 mM, Pharmacia), 2.5 U AmpliTaq (Perkin-Elmer/Cetus, Norwalk, CT) and 1×PCR buffer (Perkin-Elmer/Cetus) covered with two drops of mineral oil (Sigma). The amplification steps involved denaturation at 93°C for 1 min, annealing for 1 min at 60°C with each specific primers, and extension at 72°C for 1 min. The PCR reactions were analyzed by electrophoresis in 1.7 % agarose gels in the presence of ethidium bromide. Oligonucleotide sequences were selected with the program PRIMER (Whitehead Institute, MIT, Cambridge). The p75^{NTR}-specific primers were 5'-CGGCACCACCGACAACCTCATT (sense) and 5'-GCTCGCCTGCCAGATGTCGC (antisense), located at nt 851 and nt 1203, respectively, in the rat p75^{NTR} mRNA sequence (GenBank-EMBL accession number X05137) and amplifying a 353 bp fragment. The trkA-specific primers were 5'-ATCTGGGCAAAGCCGTGGAACA (sense) and 5'-AGGAAGAGGGCGGCGGAGACG (antisense), located at nt 933 and 1365, respectively, in the rat trkA mRNA sequence (accession number M85214) and amplifying a 433 bp fragment. The GAPDH-specific primers were 5'-CCCACGGCAAGTTCAACGG (sense) and 5'-CTTTCCAGAGGGGCCATCCA (antisense), located at nt 220 and nt 647, respectively, in the rat GAPDH mRNA sequence (accession number X02231) and amplifying a 428 bp fragment. PCR fragments were purified from the 1.7% agarose gel using the gel extraction method (Qiaex gel extraction kit, Qiagen, Chatsworth, CA) and directly sequenced by automated sequence analyzer (MediGene, Martinsried, Germany).

4.9 Flow cytometry

Purified monocytes were incubated with primary mAb or isotype-matched control antibodies as indicated for 1 h at 4°C. The cells were washed and stained with fluorescein-(DTAF)-conjugated goat anti-mouse IgG [F(ab')₂; Dianova, Hamburg, Germany] for 30 min at 4°C. The cells were then washed and immediately analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded by propidium iodide.

4.10 Culturing brain microvascular endothelial cells on Transwell™

The procedure for the isolation of rat brain microvascular endothelial cells is a modification of the technique described by Risau and colleagues [20, 28]. Cerebral cortices of 4–6 week old Lewis rats were prepared, dissected free of meninges and white matter and minced in E.H. medium. The tissue suspension was digested in 0.3% collagenase (CLSII, Seromed, Berlin, Germany) in E.H. medium for 1 h at 37°C. BSA (25%, Serva, Heidelberg, Germany) in PBS was added to the slurry and centrifugated for 20 min at 1,000×g. The pellet containing the capillary fragments was incubated in 0.1% collagenase-dispase (Boehringer Mannheim) in E.H. medium for 30 min at 37°C. After washing, the remaining pellet was incubated for 1–2 min in 10 μg /ml DNase I (Boehringer Mannheim). The capillary fragments were purified using Percoll gradient density centrifugation. The cells were then plated on six culture dishes (35 mm diameter) precoated with collagen G (Seromed). The culture medium for brain endothelial cells consisted of DMEM with 10% calf serum (Hyclone, Logan, Utah), 2.5 μg /ml amphotericin B (Seromed), 1% kanamycin, 1% nonessential amino acids, 1% sodium pyruvate (all from Gibco BRL) and 1% bovine retinal extract as a crude source of fibroblast growth factor [42]. The fragments were allowed to attach for 30 min, medium was exchanged, and the cultures were incubated at 37°C in a humidified atmosphere and 5% CO_2. Optimal purity of brain endothelial cell culture was obtained by selective lysis of contaminating astrocytes and pericytes, using anti-Thy 1.1 antibody and complement as described [28]. The confluent monolayers of the endothelial cells were dissociated by trypsinization and the single cell suspensions were used for the flow cytometric analysis or seeded onto collagen coated microporous membrane (Transwell™, Costar, Bodenheim, Germany, 6.5 mm insert diameter, 5 μm pore size). Confluency of the endothelial cell monolayer on the microporous membrane was monitored by measurement of the electrical resistance by use of EVOM™ epithelial volt-ohm-meter (World Precision Instruments, Sarasota, FL).

4.11 Monocyte transendothelial migration assay

To carry out the migration assay, the brain endothelial cells on the microporous membrane were incubated in the culture medium alone or in the presence of 10 ng/ml of TNF-α for 24 h. The monolayers were then washed twice gently and 4×10^5 of purified monocytes in 200 μl of the assay media were put into the upper chamber. The plates containing Transwell™ were incubated at 37°C, 10% CO_2 for the specified time. Following migration, each upper chamber insert was removed and migrated cells in the lower wells were transferred into a small tube. The migrated cells recovered from each lower well were sedimented, resuspended in medium and counted with a hemocytometer. The viability of the cells was assessed by trypan blue exclusion. Although the cells in the lower chamber contained some endothelial cell debris, these were easily distinguished by light microscopy so that the number of migrated cells could be accurately determined. The percentage of migrated cells was then calculated from the initial number of cells added to the upper chamber. After the assay, the endothelial cells on microporous membrane were fixed in 4% paraformaldehyde in PBS. The endothelial cell covered microporous membranes were removed from the ring with a cork borer and confluency of the endothelial cells was re-controlled by Giemsa staining. To determine the effect of neurotrophins, the monocytes adherend to dishes were subsequently treated with various concentrations of NGF for 4 h. The cells were then recovered and washed twice. 4×10^5 of neurotrophin-treated or untreated monocytes were resuspended in 200 μl of the assay media and put onto the upper chambers. The migration was measured 12 h later. Antibody blocking of NGF-induced responses was carried out using anti-p75[NTR] antiserum 9651. The monocytes were harvested from the dishes and were then treated with anti-p75[NTR] antiserum (1:100 dilution) for 1 h at 4°C. Anti-p75[NTR] antiserum treated-cells were subsequently incubated with 100 ng/ml of NGF and used for the transendothelial migration assay.

4.12 Statistical analysis

All data from the migration assay are expressed as mean ± SD of the percent migration calculated from triplicate wells. The experiments shown are representatives of three independent experiments. Statistical analysis of the data was performed using Student's t-test. p values <.01 were considered significant.

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Absence of Monocyte Chemoattractant Protein 1 in Mice Leads to Decreased Local Macrophage Recruitment and Antigen-specific T Helper Cell Type 1 Immune Response in Experimental Autoimmune Encephalomyelitis

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Abstract

Monocyte chemoattractant protein (MCP)-1 plays a critical role in innate immunity by directing the migration of monocytes into inflammatory sites. Recent data indicated a function for this chemokine in adaptive immunity as a regulator of T cell commitment to T helper cell type 2 (Th2) effector function. Studies in a Th1-dependent animal model, experimental autoimmune encephalomyelitis (EAE), showed that MCP-1 was highly expressed in the central nervous system (CNS) of affected rodents, and MCP-1 antibodies could block relapses of the disease. Mice deficient for the major MCP-1 receptor, CC chemokine receptor (CCR)2, did not develop EAE after active immunization but generated effector cells that could transfer the disease to naive wild-type recipients. We analyzed EAE in mice deficient for MCP-1 to define the relevant ligand for CCR2, which responds to murine MCP-1, MCP-2, MCP-3, and MCP-5. We found that C57BL/6 MCP-1–null mice were markedly resistant to EAE after active immunization, with drastically impaired recruitment of macrophages to the CNS, yet able to generate effector T cells that transferred severe disease to naive wild-type recipients. By contrast, adoptive transfer of primed T cells from wild-type mice into naive MCP-1–null recipients did not mediate clinical EAE. On the SJL background, disruption of the MCP-1 gene produced a milder EAE phenotype with diminished relapses that mimicked previous findings using anti–MCP-1 antibodies. There was no compensatory upregulation of MCP-2, MCP-3, or MCP-5 in MCP-1–null mice with EAE. These results indicated that MCP-1 is the major CCR2 ligand in mice with EAE, and provided an opportunity to define the role of MCP-1 in EAE. Compared with wild-type littermates, MCP-1$^{-/-}$ mice exhibited reduced expression of interferon γ in draining lymph node and CNS and increased antigen-specific immunoglobulin G1 antibody production. Taken together, these data demonstrate that MCP-1 is crucial for Th1 immune responses in EAE induction and that macrophage recruitment to the inflamed CNS target organ is required for primed T cells to execute a Th1 effector program in EAE.

Key words: autoimmune disease • chemokine • chemokine receptor • macrophage • T helper cell type 1/T helper cell type 2

Introduction

Chemokines are small proteins (8–12 kD) divided into four subfamilies (CXC, CC, C, and CX3C) according to the organization of positionally conserved cysteine residues (1). Monocyte chemoattractant protein 1 (MCP-1)[1] is a proto-

type CC chemokine, active towards monocytes, dendritic cells, and NK cells, thereby playing an important role in in-

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[1]Abbreviations used in this paper: CCR, CC chemokine receptor; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; IP-10, IFN-γ–inducible 10-kD protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MNC, mononuclear cell; MOG, myelin oligodendroglial glycoprotein; MS, multiple sclerosis; pi, postimmunization; PILN, popliteal and inguinal lymph node; PLP, proteolipid protein; RANTES, regulated upon activation, normal T cell expressed and secreted; RPA, RNase protection assay; RT, reverse transcription; TCA, T cell activation gene.

nate immunity (2–7). However, MCP-1 is also a crucial factor for the development of adaptive Th2 responses. In this regard, MCP-1 directs the differentiation of Th0 cells to Th2 in vitro (8) by a mechanism dependent on IL-4. Administration of anti–MCP-1 Abs (9) or disruption of the MCP-1 gene (2) significantly reduced the size of schistosome egg antigen (SEA) secondary granulomata, a Th2-dominant disease model. Conversely, local overexpression of MCP-1 increased the size of SEA secondary granulomata (10). Immunization with trinitrophenol-derivatized ovalbumin plus IFA elicited a reduced Th2 and unaltered Th1 response in MCP-1–deficient (MCP-1$^{-/-}$) mice. MCP-1$^{-/-}$ mice of Balb/c strain were relatively resistant to *Leishmania major* infection, indicating that lack of MCP-1 led to reduced Th2 immunity (11).

Experimental autoimmune encephalomyelitis (EAE), a model for autoimmune demyelination of the central nervous system (CNS), has been widely employed to explore pathogenic mechanisms underlying the human disease multiple sclerosis (MS [12, 13]). The generation of myelin protein–reactive T cells is an immunological hallmark of both EAE and MS and is required for disease expression in EAE. These autoreactive T cells traffic to the CNS, and initiate inflammation and destruction of CNS myelin with consequent neurological impairment (14, 15).

Th1-type T cells, producing IFN-γ, IL-2, and TNF-β, are associated with cellular immune responses, delayed-type hypersensitivity, and macrophage activation, whereas Th2-type T cells, producing IL-4, IL-5, and IL-10, are important for humoral immune responses (16, 17). The dynamic interplay and reciprocal inhibition between Th1 and Th2 cytokines has been demonstrated in numerous research reports. IL-4 is a major factor that governs Th2 differentiation and inhibits the development of IFN-γ–secreting cells (18). The activation of macrophages and the production of Th1 cytokines such as IFN-γ can also be inhibited by IL-10 (19). Most encephalitogenic T cell clones examined are Th1 polarized (20–22), although exceptions have been reported (23). Th1 cytokines are markedly elevated in the CNS of animals during EAE attacks whereas Th2 cytokines are associated with disease recovery (24). IL-4–induced immune deviation is beneficial for recovery from EAE (25); EAE can be prevented and/or reversed by myelin antigen–specific T cells that are genetically transduced with either IL-4 or IL-10 genes (26, 27); anti–IL-4 treatment reverses the tolerance induced by an altered peptide ligand (28), and absence of IL-4 in gene-targeted mice increases the severity of EAE (29). In summary, Th1 immune responses are pathogenic and Th2 responses are protective in the initiation and evolution of EAE.

However, antigen-specific T cells constitute only a small proportion of infiltrating leukocytes in EAE or MS lesions (30). Secondarily recruited inflammatory cells account for the vast majority of infiltrating cells and play a pivotal role in CNS tissue damage (31). Although the detailed mechanisms by which inflammatory cells influx into the CNS compartment are not completely understood, increasing evidence suggests that chemokines, in concert with adhesion molecules, are essential for this process (32). In EAE, elevated expression of MCP-1 by CNS parenchymal cells, tightly linked to clinical disease, has been demonstrated repeatedly (33–35). Further, anti–MCP-1 Abs blocked relapses of adoptive transfer EAE in SJL mice (36). Additionally, mice that lacked CC chemokine receptor 2 (CCR2), the major receptor on monocytes for MCP-1, failed to develop EAE after active immunization and were resistant to induction of EAE by the adoptive transfer of primed T cells from syngeneic wild-type mice (37, 38). It was uncertain whether MCP-1 was the relevant ligand for CCR2 in these experiments, as this receptor also responds to MCP-2, MCP-3, and MCP-5. However, there is also support for the possibility that regulation of Th2 responses by MCP-1 could be important for the pathogenesis of EAE; in particular, MCP-1 was critical for the development of tolerance after oral administration of a proteolipid protein (PLP) peptide containing residues 139–151 (39)

Therefore, the phenotype of EAE in MCP-1–deficient mice could not readily be predicted. On one hand, defective MCP-1–dependent monocyte recruitment might lead to attenuated disease. Alternatively, if functional replacement of MCP-1 by another MCP mediated monocyte accumulation in the CNS of these mice, defective Th2 responses might lead to very severe, nonremitting disease. Finally, in view of redundancy in the immune/inflammatory system, it remained possible that MCP-1–null mice would manifest EAE identically to wild-type animals. Given these considerations, the role of MCP-1 in the pathogenesis of EAE merited further characterization.

These concerns are also likely pertinent for the human disorder MS. Patients with active disease, manifest by clinical attacks, showed significantly decreased MCP-1 in the cerebrospinal fluid, as compared with controls. Of eight chemokines measured, only MCP-1 was reduced in the cerebrospinal fluid of patients with active MS. However, abundant MCP-1 has been readily detected by immunohistochemistry in autopsy brain sections containing MS lesions (40–42). Therefore, the role of MCP-1 in the pathogenesis of MS remains to be clarified.

In this report, we describe the phenotype of EAE in MCP-1–null mice. These mice exhibited markedly reduced clinical and histological EAE after active immunization and did not develop clinical disease after receiving encephalitogenic T cells from wild-type animals. Expressions of MCP-2, MCP-3, and MCP-5 in the CNS of both wild-type and MCP-1–null mice with EAE were virtually identical. These findings indicated that MCP-1 was the major ligand for CCR2 in murine EAE. In this EAE model, we found that disruption of the MCP-1 gene led to an attenuated Th1 autoimmune response and complimentarily increased Th2 response. These results indicated a crucial role for MCP-1 in generating CNS inflammatory reactions that mediate the effector phase of myelin-specific Th1 autoimmune responses. Therefore, the data suggested that primed encephalitogenic Th1 cells cannot manifest effector functions in the CNS without recruiting hematogenous macrophages.

Materials and Methods

Mice. The disruption of the MCP-1 gene has been described previously (2). MCP-1$^{-/-}$ mice were backcrossed onto the C57BL/6 (B6) strain for eight generations. One F8 MCP-1$^{-/-}$ mouse was further backcrossed to a B6 mouse (obtained from The Jackson Laboratory). The heterozygous offspring were intercrossed to produce F9 wild-type (+/+), heterozygous (−/+), and MCP-1$^{-/-}$ mice. F10 mice were generated in a similar manner. MCP-1$^{+/+}$, MCP-1$^{-/+}$, and MCP-1$^{-/-}$ F9 and F10 mice on the B6 background were used in this study.

MCP-1-deficient mice on the B6/129 background were also backcrossed onto SJL for seven generations. MCP-1$^{+/+}$ and MCP-1$^{-/-}$ F7 mice on SJL background were used in this study.

Mice were genotyped using a PCR-based analysis of genomic DNA extracted from tail clips. Primers MCP-1F, 5′-GGA GCA TCC ACG TGT TGG C-3′ and MCP-1R, 5′-ACA GCT TCT TTG GGA CAC C-3′ amplified a DNA fragment within the MCP-1 gene. Primers NeoF, 5′-CGC TTC CTT TTT GTC AAG AC-3′ and NeoR, 5′-ATC CTC GCC GTC GGG CAT GC-3′ amplified a fragment in the neomycin resistance gene insert. PCR reactions were performed in a PerkinElmer 9700 cycler (annealing temperature, 50°C) and products were visualized by electrophoresis on ethidium bromide–stained NuSeive GTG® agarose gel.

Rat Myelin Oligodendroglial Glycoprotein and Mouse Proteolipid Protein Peptides. Rat myelin oligodendroglial glycoprotein (MOG)35–55 and mouse PLP139–151 peptides were obtained from (BIO·SYNTHESIS) and purified by HPLC with a purity of 98%. The sequence of MOG35–55 was MEVGWYRSPFS-RVVHLYRNGK and that of PLP139–151 was HSLGKWL-GHPDKF.

Active Induction of EAE with MOG and PLP Peptides and Clinical Evaluation. Mice of 8–9 wk of age were subcutaneously injected with 300 µg MOG35–55 emulsified in CFA (Difco) containing 400 µg *Mycobacterium tuberculosis.* Mice were intravenously injected with pertussis toxin (Sigma-Aldrich) as indicated in the figure legends on day 0 and 2 postimmunization (pi). The immunization in SJL mice was carried out as described previously (43). All mice were weighed, examined, and graded daily for neurological signs in a double blind manner by one of us (J. Wang) as follows: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; and 5, moribund state. Disease relapse was determined when an increase of one EAE score unit was observed. Signs of neurological impairment were typically accompanied by an abrupt, substantial weight loss (>7%). The average day of EAE onset was calculated by adding the first day of clinical signs for individual mice and divided by the number of mice in the group. Day of EAE onset in mice that showed no clinical EAE was deliberately regarded as 1 d after the experiment was terminated (44). The EAE index was calculated by adding all the daily EAE scores to obtain cumulative score and dividing by day of EAE onset. Active immunization with MOG35–55 induced monphasic EAE in B6 mice and was followed for 65 d. Chronic relapsing EAE induced by PLP139–151 was monitored for 90 d.

T Cell Proliferation Assay. Mice were killed and draining lymph nodes (popliteal and inguinal lymph nodes [PILNs]) were dissected on day 10 pi. Single cell suspensions (5 × 10^6/ml) were prepared and cultured in triplicate in 96-well flat-bottomed plates (Falcon; Becton Dickinson) in 200 µl/well in the presence or absence of MOG35–55, PLP139–151, LPS, or anti-CD3 (R&D Systems) in RPMI 1640 (GIBCO BRL) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ ml streptomycin, 10% FCS, and 5.5 × 10^{-5} M 2-mercaptoethanol. Cells were pulsed with [^{3}H]thymidine (Amersham Pharmacia Biotech), 0.5 µCi/well 72 h after culture initiation, and incubated 10 h further. Plates were harvested using a harvester (INOTECH). Incorporated radioactivity was measured in a MicroBeta PLUS liquid scintillation counter with software v3.3 (Wallac Co.).

Cell Cultures and Cytokine Assay. Mononuclear cell (MNC). suspensions (5 × 10^6/ml) were prepared from PILNs of mice that had been immunized with MOG35–55 plus CFA for 10 d. Cells were cultured at 2 × 10^6/ml in RPMI 1640 supplemented as above in cell culture tubes (Falcon; Becton Dickinson) in the presence or absence of MOG35–55 and anti-CD3ε Ab at 5% CO$_2$ and 95% humidity. The supernatants were collected after 24, 48, and 72 h of in vitro restimulation and kept at −80°C until assay. Levels of IFN-γ, IL-4, and IL-10 in sera and cell culture supernatants were determined using ELISA kits commercially obtained from R&D Systems. The standard curves were made on the same occasion and the sensitivities for the methods were 2.0, 2.0, and 4.0 pg/ml for IFN-γ, IL-4, and IL-10, respectively. All samples were measured in duplicate and diluted if necessary.

Analysis of Chemokine and Chemokine Receptor mRNA Levels by RNase Protection Assay. Mice were anesthetized with sodium pentobarbital and intracardially perfused through the left ventricle with ice-cold PBS. Spinal cords were extruded by flushing the vertebral canal with PBS, rinsed in PBS, and kept at −80°C. Total cellular RNA was prepared from spinal cord tissue by TRIzol (Life Technologies). Quantification of CC chemokines and chemokine receptors in CNS tissue was done by RNase protection assay (RPA) with Template Sets and In vitro Transcription Kit (BD PharMingen) according to the manufacturer's instructions. Protected fragments were visualized and quantified by autoradiography with PhosphorImager (Molecular Dynamics).

Reverse Transcription PCR Detection for Levels of MCPs, Cytokine, CD3ε, CD8, and Mb-1 mRNA. Total RNA was extracted from the PBS-perfused, snap-frozen spinal cords using TRIzol. cDNA was synthesized using a RNA PCR Core kit (Gene-Amp®; PerkinElmer). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an unregulated control and amplified using primers: GAPDHf, 5′-GGT GGA GGT CGG AGT CAA CG-3′ and GAPDHr, 5′-CAA AGT TGT CAT GGA TGA CC-3′. MCP-2, MCP-3, and MCP-5 cDNA were amplified by specific primer pairs, mcp-2f, 5′-ACA TCA CCT GCT TGG TCT GGA AAA C-3′ and mcp-2r, 5′-ACT AAA GCT GAA GAT CCC CCT TCG-3′; mcp-3f, 5′-CAC ATT CCT ACA GAC AGC TC-3′ and mcp-3r, 5′-AGC TAC AGA AGG ATC ACC AG-3′; and mcp-5f, 5′-CTC CTT ATC CAG TAT GGT CC-3′ and mcp-5r, 5′-TCT CCC TCC ACC ATG CAG AG-3′. IFN-γ, IL-4, and IL-10 were amplified with primer pairs: IFNgf, 5′-AGC GGC TGA CTG AAC TCA GAT TGT-3′ and IFNgr, 5′-GTC ACA GTT TTC AGC TGT ATA GGG-3′; il4f, 5′-TCG GCA TTT TGA ACG AGG TC-3′ and il4r, 5′-GAA AAG CCC GAA AGA GTC TC-3′; and il10f, 5′-CAT CAT GTA TGC TTC TAT GC-3′ and il10r, 5′-TAC CTG GTA GAA GTG ATG CC-3′. CD3ε was detected with primer pair: CD3ef, 5′-ATG GAG CAG AGG AAG GGT CTG-3′ and CD3er, 5′-TCA CTT CTT CCT CAG TTG GTT-3′. Levels of CD8 cDNA were detected using primer pair: CD8f, 5′-TCT GTC GTG CCA GTC CTT C-3′ and CD8r, 5′-CCT TCC TGT CTG ACT AGC GG-3′, while that of Mb-1 (the gene encoding IgGα, expressed by B cells; reference 45): Mb-1f, 5′-GCC AGG GGG TCT AGA AGC-3′ and Mb-1r,

5'-TCA CTT GGC ACC CAG TAC AA-3'. RNA Amplification Kit SYBR Green I was used in all PCR reactions that were performed in a real-time LightCycler system (Roche Molecular Biochemicals). The level of specific mRNA was quantified and expressed as the cycle number at which the LightCycler System detected the upstroke of the exponential phase of PCR product accumulation, and normalized by the level of GAPDH expression in each individual sample. Therefore, levels of input mRNAs correlate negatively with numbers of cycles.

Determination of Serum Anti–MOG35–55 IgG, IgG1, and IgG2a. Detection of anti–MOG35–55 Abs was performed as described previously (46). In brief, rat MOG35–55 peptide (0.1 ml/well, 3.0 µg/ml) was added to 96-well microtiter plate (Nunc) in coating buffer and incubated at +4°C overnight. After being washed three times with PBS-Tween (0.1%), the plates were blocked with 5% FCS in PBS for 2 h at room temperature. Diluted sera (0.1 ml/well, 1:5 for IgG2a, 1:50 for total IgG and IgG1) were added to and incubated at room temperature for 2 h, followed by washing with PBS-Tween three times. A series of serum dilutions were examined in the preliminary experiments. The OD values obtained negatively correlated with the dilutions of the samples. For the detection of IgG2a, alkaline phosphatase–conjugated anti–mouse IgG2a (1:2,000 dilution; BD PharMingen) was added and incubated for 2 h at room temperature, followed by five washes. Color was developed by adding *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) and measurements were performed at 450 nm in an ELISA reader (Wallac Co.). For the detection of total IgG and IgG1, biotinylated anti–mouse IgG (1:3,000; Sigma-Aldrich) and IgG1 (1:500; BD PharMingen) Abs were added and incubated at room temperature for 2 h. After incubation with ABC Vectastain (Vector Laboratories), the reaction was developed with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) peroxidase substrate (Vector Laboratories) and read at 405 nm in the ELISA reader. In each assay for IgG, IgG1, or IgG2a, all the samples were measured in duplicate on a single plate.

Flow Cytometry. Cells from the CNS were isolated as described previously (37). Samples were washed in FACS® buffer (1% FCS and 0.1% sodium azide in PBS). After blocking with CD16/CD32 Fc Block (BD PharMingen), cells were stained for surface markers with directly conjugated Abs in FACS® buffer. Abs used were CD4–FITC, CD11b–PE, CD45–Cy-Chrome, and CD8–Cy-Chrome, and were titrated using mouse peripheral blood. All Abs were obtained from BD PharMingen.

Adoptive Transfer of EAE. Mice were immunized with 200 µg MOG35–55 peptide in CFA. Lymph nodes were harvested and single MNC suspensions were prepared 10 d pi and cultured in the presence of 30 µg/ml MOG35–55 and 20 ng/ml murine rIL-12 (R&D Systems). After 4 d incubation, cells were collected and washed in PBS. 2×10^7 living cells were intravenously injected into wild-type or MCP-1–deficient mice. Recipients were given 200 ng pertussis toxin intravenously on the day of cell transfer and 48 h after transfer (37, 47). Mice were weighed and EAE scored daily by an investigator (J. Wang) who was blinded to mouse genotype or source of transferred cells. Mice were monitored for 40 d after transfer.

Statistical Analyses. The Instat 2.02 software was used for the analyses of the difference between the MCP-1+/+, MCP-1–/+, and MCP-1–/– mice. The Mann-Whitney U-test was used for the comparisons of disease severity and cytokine and chemokine gene expressions. A chi-square test was used for the comparisons of disease incidence in groups of MCP-1+/+, MCP-1–/+, and MCP-1–/– mice. A *P* value <0.05 was considered as significant.

Results

C57BL/6 MCP-1–deficient Mice Are Relatively Resistant to Active EAE Induction with MOG35–55.

MCP-1+/+, MCP-1–/+, and MCP-1–/– F9 mice (*n* = 9 in each group) were immunized with 300 µg MOG35–55 plus 500 ng pertussis toxin intravenous injection on day 0 and 2 pi. One MCP-1–/– mouse died of immunization on day 4 pi. The remainder of the mice, regardless of genotype, developed clinical EAE. MCP-1+/+ mice showed EAE signs around day 10 pi, consistent with previously reported results in C57BL/6 mice (44, 48). MCP-1–/– manifested significantly delayed EAE with an average onset on day 21 pi (Fig. 1). Three out of nine wild-type mice died of EAE, and another three had to be killed because of moribund state. None of the MCP-1–/– mice died of EAE or had to be killed throughout the experiment and they recovered from the disease significantly faster and more completely than wild-type littermate controls. Heterozygote MCP-1–/+ mice developed EAE with an intermediate kinetics and severity. None of the MCP-1–/+ mice died of EAE, whereas three were killed in a moribund state due to severe EAE. Analyses of CNS tissue histology revealed massive inflammatory infiltrates in wild-type control mice (+++ to ++++ in regions of lumbar and sacral spinal cord, *n* = 4; Fig. 2 A) but markedly reduced inflammatory reaction in MCP-1–null mice (+ to ++ in regions of corresponding levels of affected spinal cord, *n* = 4; Fig. 2 B; reference 49). Examination of demyelination using Luxol Fast Blue stain-
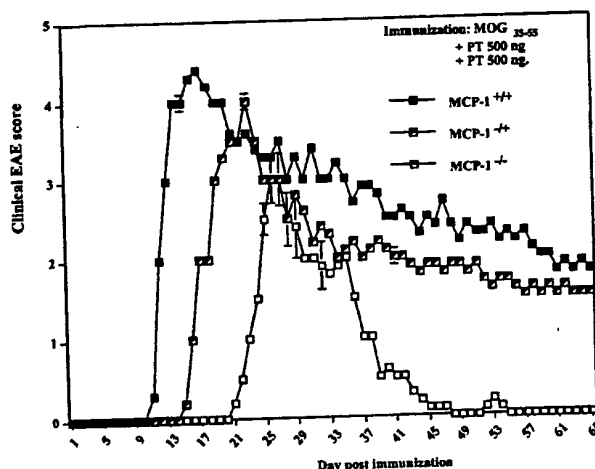

Figure 1. Effect of MCP-1 gene disruption on MOG35–55–induced EAE. F9 MCP-1+/+, MCP-1–/+, and MCP-1–/– mice were immunized with MOG35–55 emulsified in CFA and intravenously injected with pertussis toxin (PT) on the day of immunization and 48 h later (500 ng/injection). Three wild-type mice died of EAE and another three had to be killed due to severe EAE attack. None of the MCP-1–/+ mice died of EAE, but three were killed. None of the MCP-1–/– mice died of EAE or had to be killed. Shown are EAE score (mean ± SD) in each group of mice that had been followed throughout the experiment (*n* = 3, 6, and 8 for MCP-1+/+, MCP-1–/+, and MCP-1–/– group, respectively). This graph is representative of three experiments with similar results.
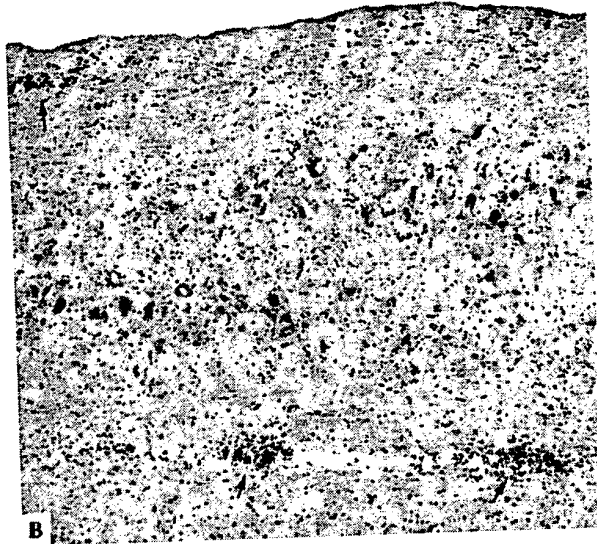
Figure 2. Spinal cord histology of MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice with EAE score 4.0. Hematoxylin and eosin staining of longitudinal cryosections. Note the numerous perivascular cuffs and subpial infiltrates (arrows) as well as leukocytes disseminated in the white matter of MCP-1$^{+/+}$ mice (A), whereas merely fewer inflammatory infiltrates were found in MCP-1–deficient mice (B).

Figure 3. Attenuated MOG35–55–induced EAE in MCP-1–null mice. F10 MCP-1$^{-/-}$ mice ($n = 6$) and their littermate wild-type controls ($n = 6$) were immunized with MOG35–55 in CFA and intravenously injected with pertussis toxin (PT; 200 ng/injection). Shown are EAE score (mean ± SD) of individuals in each group.

ing revealed significant reduction in MCP-1$^{-/-}$ mice compared with their littermates controls 65 d after immunization (data not shown).

F10 MCP-1–deficient mice and wild-type littermate controls were analyzed in a subsequent experiment. As pertussis toxin has been shown to increase the permeability of the blood-brain barrier (BBB [50, 51]), enhance delayed type hypersensitivity (DTH) responses and the production of IFN-γ (52–54), augment expression of CD80, CD86 on antigen-presenting cells and CD28 on T cells (55), and
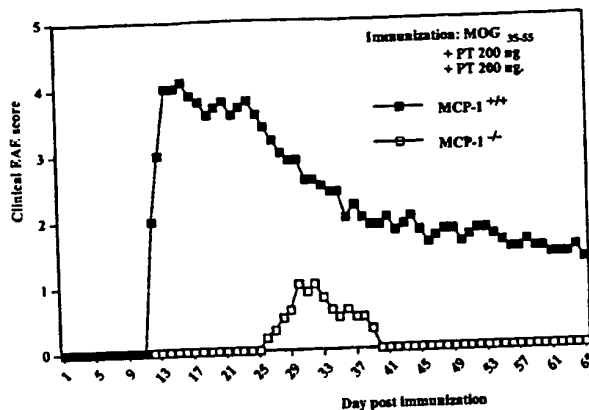
T cell immune responses (55, 56), we reduced the amount of pertussis toxin in the immunization to 200 ng per injection in an attempt to reduce the high death rate observed in wild-type mice in the F9 experiments. In this experiment, all MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice showed signs of clinical EAE; none died of EAE or required killing. In contrast to the MCP-1$^{+/+}$ mice that developed full-blown EAE, the disease was largely suppressed with significantly delayed onset and milder neurological impairment (Fig. 3) and significantly less weight loss (data not shown) in MCP-1–deficient mice. These data demonstrated that MCP-1–null mice and CCR2-deficient mice exhibited strikingly similar EAE phenotypes (37, 38) and suggested that MCP-1 may be the relevant ligand for CCR2 in this model.

Disruption of MCP-1 Gene Attenuates the Severity of PLP-induced EAE and Reduces the Number of Relapses in SJL Mice. 11 MCP-1$^{+/+}$ and 10 MCP-1$^{-/-}$ mice on SJL background were immunized with PLP139–151 peptide emulsified in CFA. One MCP-1$^{+/+}$ and two MCP-1$^{-/-}$ mice died of immunization within the first week after immunization. The reminder of MCP-1$^{+/+}$ mice (10/10) and 7 out of 8 MCP-1$^{-/-}$ mice showed clinical EAE. Compared with the MCP-1$^{-/-}$ group, a significantly higher percentage of MCP-1$^{+/+}$ mice died of EAE during the first attack and relapses thereafter. Among the mice that survived the first attack, six MCP-1$^{+/+}$ mice had eight relapses (four mice had one attack each and two had two attacks each), whereas three out of seven MCP-1$^{-/-}$ mice had single attacks. At day 90 pi, the average EAE index was significantly higher in MCP-1$^{+/+}$ group ($n = 4$) than that in MCP-1$^{-/-}$ group ($n = 7$) (Fig. 4). These results implicated a role for MCP-1 in eliciting relapses of EAE in this model. These findings were consistent with previous reports showing reduction of relapses using anti–MCP-1 Abs in a passive EAE model (36) and in mice receiving vaccine containing naked DNA encoding for MCP-1 (57).
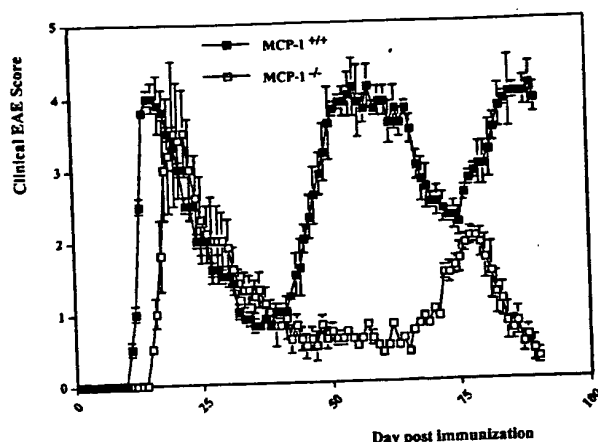
Figure 4. Milder disease and reduced number of relapses in PLP-induced EAE MCP-1$^{-/-}$ SJL mice. MCP-1$^{-/-}$ SJL mice and their litter-mate wild-type controls were immunized with PLP139–151 in CFA plus intravenous injection of pertussis toxin as described in Materials and Methods. Mice were monitored for 90 d after immunization. MCP-1$^{-/-}$ SJL mice showed significantly decreased EAE index, reduced number of relapses, and nonsignificantly delayed disease onset.

There Is No Compensatory Upregulation of MCP-2, MCP-3, or MCP-5 in the CNS of MCP-1$^{-/-}$ Mice with EAE. Because CCR2 is shared in common among all MCPs (58–60), we analyzed MCP-2, MCP-3, and MCP-5 mRNA expression in the CNS of C57BL/6 mice with MOG-induced EAE, using quantitative real-time reverse transcription (RT)-PCR. Levels of MCP-2 and MCP-3 but not MCP-5 were elevated in CNS tissue from EAE mice. There was no significant difference in MCP-2 expression in CNS tissue between MCP-1$^{+/+}$ and MCP-1$^{-/-}$ group (23.8 ± 0.4 vs. 23.7 ± 0.5, mean ± SD; $n = 5$ in each group). No significant difference of MCP-3 expression in CNS tissue from MCP-1$^{+/+}$ (31.0 ± 0.6, $n = 5$) and MCP-1$^{-/-}$ (33.1 ± 2.6, $n = 5$, $P = 0.4$) mice was found, whereas CNS MCP-5 expression in mice with EAE was essentially undetectable (data not shown). The unaltered levels of MCP-2 and MCP-5 and a trend towards decreased expression of MCP-3 in MCP-1$^{-/-}$ mice (higher PCR cycle number) uncovered no compensatory expression of other MCPs in this system, consistent with a previous report in autoimmune kidney disease model in MCP-1$^{-/-}$ MRL-*Fas*^{lpr} mice (61). These findings supported the hypothesis that the similarity of EAE phenotype in MCP-1– and CCR2-deficient mice is caused by absence of the ligand, i.e., MCP-1 signaling pathway through its major receptor CCR2.

T Cell Proliferation to MOG35–55 Peptide. To examine the afferent limb of the immune response to MOG peptide in wild-type and MCP-1–null mice, MNC suspensions were prepared from PILNs primed with 200 μg MOG35–55 peptide in CFA and rechallenged with MOG35–55 in vitro. MCP-1–deficient mice showed a nonsignificantly higher recall T cell response than wild-type controls. No T cell recall response was induced by stimulation with
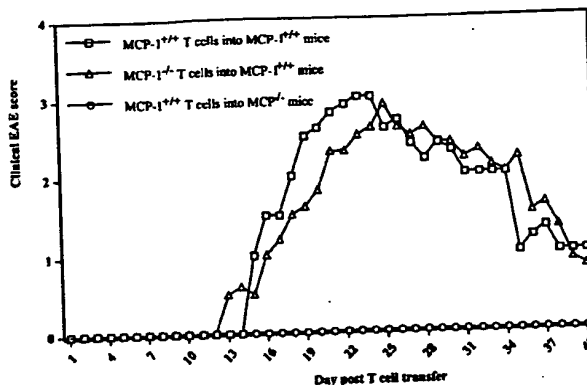
PLP139–151 in vitro, a specificity control. No difference in anti-CD3ε–induced T cell proliferation was found between wild-type and MCP-1–deficient mice (data not shown). These data indicated that the CD3 pathway was intact in MCP-1$^{-/-}$ mice, and that MOG35–55–specific T cells can be generated in MCP-1–deficient mice.

MCP-1$^{-/-}$ Mice Do Not Develop Clinical EAE in Passive Transfer Model. An adoptive transfer EAE model was used to further address whether MCP-1–null mice could develop pathogenic autoimmune responses to MOG35–55 peptide. MCP-1$^{+/+}$ and MCP-1$^{-/-}$ B6 mice were immunized with MOG35–55 peptide in CFA, and MNCs isolated from draining lymph nodes were cultured in the presence of MOG35–55 peptide and IL-12 before transfer into MCP-1$^{-/-}$ mice or littermate controls. As expected, MCP-1$^{+/+}$ mice receiving MCP-1$^{+/+}$ T cells developed clinical EAE (Fig. 5). MCP-1$^{-/-}$ T cells showed approximately the same encephalitogenic capacity in this adoptive transfer model, resulting in a comparable incidence, severity, and clinical course of EAE in MCP-1$^{+/+}$ recipients. In contrast, MCP-1$^{-/-}$ mice that received MCP-1$^{+/+}$ T cells failed to develop clinical EAE (Fig. 5). This result indicated that the attenuated EAE in MCP-1$^{-/-}$ mice was not caused by impaired generation of encephalitogenic T cells. The data also demonstrated that absence of MCP-1 expression in the recipient rendered the mice unable to respond to encephalitogenic signals produced by wild-type T cells.

To dissect the mechanisms underlying the relatively resistance to EAE induction in MCP-1–deficient mice, the MOG35–55–induced EAE model was used in the following mechanistic studies. To obtain samples from MCP-1$^{-/-}$ mice with full-blown EAE, F10 mice were immunized with MOG35-55 in CFA plus 500 ng pertussis toxin per injection.

Reduced CD11b$^+$CD4$^-$/CD4$^+$ Ratio but Unchanged Levels of CD3ε and CD8 Transcripts in CNS Tissue from MCP-1$^{-/-}$ Mice with EAE. Results described above suggested that MCP-1–null mice were deficient in recruiting mono-

Figure 5. Clinical course of EAE in MCP-1$^{-/-}$ and littermate control recipient mice that received MOG35–55–reactive T cells generated from MCP-1$^{+/+}$ or MCP-1$^{-/-}$ littermates. Data are presented as the EAE score (mean ± SD) in each group. Each group consists of four recipient mice.

cytes to the CNS during EAE. To address this issue, leukocytes were isolated from CNS tissue of wild-type and MCP-1–null mice with comparable severity of EAE, and analyzed with flow cytometry. Cell numbers in preparations isolated from MCP-1$^{-/-}$ mice during EAE attacks (score 4) were ~1/3 of those from MCP-1$^{+/+}$ littermate controls with comparable EAE severity. Compared with MCP-1$^{+/+}$ littermate controls, MCP-1$^{-/-}$ mice showed a sharply reduced percentage of CD11b$^+$CD4$^-$ cells in the CNS during EAE attacks (71.8 ± 4.6% vs. 44.0 ± 4.2%, mean ± SD, $n = 5$ and 4, respectively; $P < 0.0001$). In contrast, percentages of CD4$^+$ cells were relatively increased in MCP-1$^{-/-}$ mice (20.2 ± 5.9% vs. 39.8 ± 2.7%, mean ± SD, $n = 6$ and 4, respectively; $P < 0.001$; Fig. 6). The CD4$^+$ infiltrating T cells expressed high levels of CD11b ($\alpha_M\beta_2$ integrin) both in MCP-1$^{+/+}$ (79.0 ± 5.6, $n = 5$) and MCP-1$^{-/-}$ (78.1 ± 6.8, $n = 5$) mice, indicating that most infiltrating CD4$^+$ T cells are activated.

To normalize percentage of CD4$^+$ T cells in the CNS infiltrates of wild-type and MCP-1–null mice with EAE, total CNS T cells were analyzed by determining levels of CD3ϵ in the CNS. We found no significant difference in CNS CD3ϵ mRNA levels between wild-type (0.789, $n =$

5; CD3ϵ/GAPDH) and MCP-1$^{-/-}$ (0.791, $n = 5$; CD3ϵ/GAPDH) mice with clinical EAE scores of 3.5–4.0, suggesting that total T cell numbers in the CNS of wild-type and MCP-1–null mice with EAE were equivalent. Therefore, the increased proportion of CD4$^+$ cells in the CNS leukocyte infiltrates of MCP-1–null mice was caused by a marked reduction in the number of CD11b$^+$CD4$^-$ cells in the CNS of MCP-1–deficient mice with EAE.

CD8$^+$ T cells play an important downregulatory role in the pathogenesis of EAE (62). The possibility that the milder clinical EAE phenotype observed in MCP-1$^{-/-}$ mice might be due to an increased number of CD8$^+$ T cells in the CNS infiltrates was unlikely based on the fact that levels of CD8-specific mRNA were virtually identical in MCP-1$^{-/-}$ (24.08 ± 0.3, $n = 5$) and MCP-1$^{+/+}$ (24.21 ± 0.2, $n = 5$) CNS tissue during EAE attack. The passive transfer EAE model was used to examine further if CD8$^+$ T cells can be preferentially recruited into CNS in the absence of MCP-1. MOG35–55–reactive MCP-1$^{+/+}$ T cells were incubated in the presence of MOG35–55 and IL-12, and were injected intravenously into MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice. To reduce the influence of secondarily recruited macrophages in the CNS, recipient mice were killed at day 3 and 4 after T cell transfer, before the onset of clinical EAE. CNS-infiltrating T cells were recovered and analyzed using flow cytometry. No difference was found between MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice (data not shown). These results demonstrated that disruption of MCP-1 gene exerts no significant impact on the recruitment of adoptively transferred T cells into inflammatory CNS tissue. No significant difference in Mb-1 mRNA levels was found in EAE-affected CNS tissue from MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice (data not shown).

We also examined the leukocyte infiltrates in MCP-1$^{-/-}$ mice at day 14 pi when the MCP-1$^{+/+}$ controls were undergoing EAE attacks while the MCP-1$^{-/-}$ mice were still free of EAE signs. Shown in Fig. 6 C are numerous CD45^{high}CD11b$^+$ (mainly containing macrophages/activated microglia and activated T cells) isolated from MCP-1$^{+/+}$ mice during EAE attack on day 14 pi. In contrast, the majority of cells isolated from MCP-1$^{-/-}$ mice were CD45^{low}CD11b$^+$ microglia, and the components of the infiltrates (Fig. 6 D) were virtually the same as those from healthy unimmunized mice (data not shown).

Diminished MOG35–55–specific Th1 Cytokine Responses in MCP-1–null Mice. Significant changes in cytokine production have been described in the Th1 immune response that typifies MOG35–55 peptide–induced EAE in B6 mice (35). Serum concentrations of IFN-γ, IL-4, and IL-10 were determined by ELISA from mice immunized with MOG35–55 on days 8 and 10 pi and at the peak of EAE (day 14 pi in wild-type controls and 25 pi in MCP-1–deficient mice). At day 8 pi, concentrations of IFN-γ were slightly but significantly higher in wild-type than in MCP-1–deficient mice. This difference between wild-type and MCP-1–deficient mice became strikingly evident on day 10 pi near the onset of EAE in wild-type controls. The onset of EAE was also associated with increased serum levels
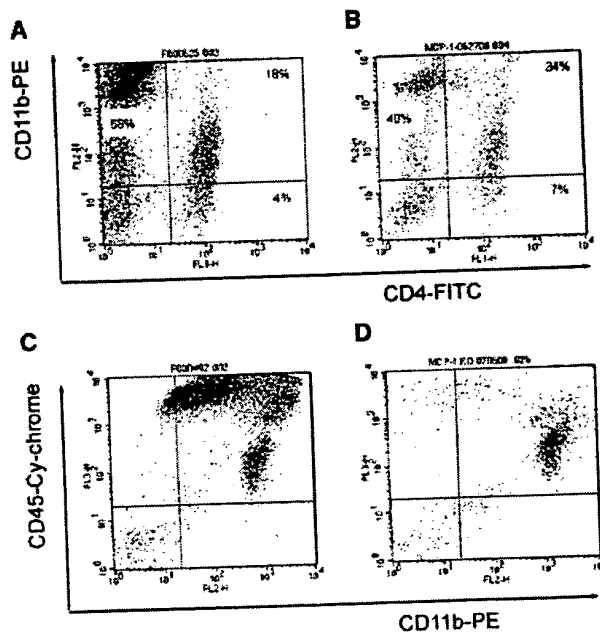

Figure 6. Altered pattern of CNS cell infiltrates in MCP-1–null mice. MCP-1$^{-/-}$ and MCP-1$^{+/+}$ littermate controls were immunized with MOG35–55 and pertussis toxin (500 ng/injection) and killed at the peak of EAE (score 4). Cells were isolated from the CNS and stained with anti-CD4–FITC, anti-CD11b–PE, and anti-CD45–Cy mAbs. Compared with wild-type controls (A), the percentages of CD11b$^+$CD4$^-$ cells were significantly decreased whereas the percentages of CD4$^+$ T cells increased in MCP-1$^{-/-}$ mice (B). In contrast to MCP-1$^{+/+}$ mice that developed in MCP-1$^{-/-}$ mice (B). In contrast to MCP-1$^{+/+}$ mice that developed full-blown EAE with numerous CNS CD11b$^+$CD45^{high} macrophages/ activated microglia and T cells (C), the majority of cells isolated from CNS tissues of EAE symptom-free MCP-1$^{-/-}$ mice on day 14 pi were CD11b$^+$CD45^{low} microglia (D).
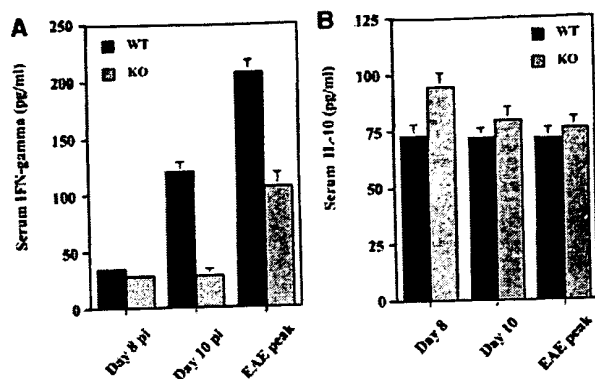
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Figure 7. Quantitation of circulating cytokine levels in MCP-1$^{-/-}$ and MCP-1$^{+/+}$ mice immunized with MOG35–55 in CFA and pertussis toxin (500 ng/injection). Sera were collected at different times after immunization and measured for levels of IFN-γ (A), IL-10 (B), and IL-4 (undetectable, data not shown). Data are mean ± SD of five individual mice in each group. This histogram is representative of two experiments with similar results. WT, wild-type; KO, knockout.

Figure 8. Cytokine levels in cell culture supernatants of lymphocytes from MOG35–55–immunized MCP-1–deficient mice and their littermate controls. Cells from draining lymph nodes were restimulated with MOG35–55 peptide in vitro. Supernatants were collected at different times after immunization as indicated and measured for IFN-γ, IL-10, and IL-4 (undetectable, data not shown). Data represent means ± SD and are representative of two experiments with similar results. WT, wild-type; KO, knockout.

of IFN-γ in MCP-1–deficient mice, but the magnitude of increase was significantly less than in MCP-1$^{+/+}$ mice (Fig. 7 A). Circulating IL-10 was detected at low levels in both MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice before and after the onset of EAE. Before EAE onset at day 8 pi (Fig. 7 B), serum IL-10 was slightly but significantly higher in MCP-1$^{-/-}$ mice than in wild-type controls. Serum IL-4 remained below the limits of detection in both MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice at all time points.

These results were supported by data from in vitro restimulation experiments, using draining lymph node (PILN) cells from mice immunized with MOG35–55 in CFA. Upon rechallenge with MOG35–55 peptide in vitro, at all examined occasions during the cell culture, PILN cells from MCP-1$^{-/-}$ mice secreted ~50% less IFN-γ than cells from MCP-1$^{+/+}$ mice (Fig. 8, left). IL-4 and IL-10, signature Th2 cytokines in EAE (26, 29, 63, 64), were also measured. Although low levels of IL-10 were found in the culture supernatants of restimulated PILN cells from both MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice, significantly higher levels of IL-10 were detected in cultures of cells from MCP-1$^{-/-}$ mice (Fig. 8, right). IL-4 was undetectable in all cell culture supernatants.

CNS Cytokine mRNA Accumulation in MCP-1$^{-/-}$ and Wild-type Mice with EAE. Local expression of IFN-γ in the CNS was analyzed by real-time RT-PCR. Significantly higher geometric mean levels of IFN-γ were found in spinal cord tissue from MCP-1$^{+/+}$ mice at the peak of EAE attacks (MCP-1$^{+/+}$: 27.7 ± 0.3, mean ± SD, n = 4; MCP-1$^{-/-}$: 29.9 ± 0.8, n = 4, P < 0.05). This result indicated approximately a fourfold difference in the CNS expression of IFN-γ between mice with intact and disrupted MCP-1 genes, despite equal numbers of CNS-infiltrating T cells (see above). There was no difference in IL-10 expression in CNS tissue between MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice with full-blown EAE. IL-4 gene expression was un-
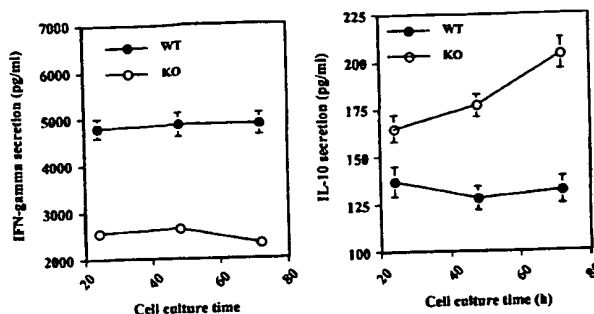
detectable both in MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice (data not shown).

Decreased Expression of IFN-γ–inducible 10-kD Protein, Macrophage Inflammatory Protein 1α, and RANTES in CNS Tissue from MCP-1$^{-/-}$ Mice with EAE. CNS chemokine expression was quantified using RPA, in tissues from MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice equally affected by EAE (score 3.5–4.0). MCP-1$^{-/-}$ mice had significantly lower levels of IFN-γ–inducible 10-kD protein (IP-10), macrophage inflammatory protein (MIP)-1α, and regulated upon activation, normal T cell expressed and secreted (RANTES) transcripts compared with wild-type littermate controls (Fig. 9). Expression of MCP-3 was low in MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice at the peak of EAE attack without significant differences between MCP-1–deficient mice and littermate controls, supporting the results obtained using real-time RT-PCR. T cell activation gene (TCA)-3 expression in the CNS of MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice with EAE was near the lower limits of detection. No significant difference was found in CCR gene expression (data not shown). However, there was a nonsignificant trend towards decreased levels of CCR2 expression in MCP-1$^{-/-}$ EAE CNS tissue compared with wild-type controls. In the ab-
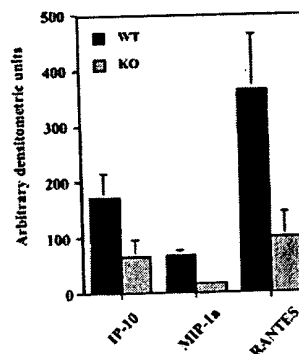

Figure 9. Chemokine expression in spinal cords from MCP-1$^{-/-}$ and MCP-1$^{+/+}$ mice immunized with MOG35–55 and pertussis toxin (500 ng/injection). Spinal cords were collected from mice that were equally affected by EAE (score 4) and levels of chemokine mRNA were measured using RPA. Data are presented as mean chemokine products ± SD. WT, wild-type; KO, knockout.

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sence of MCP-1, the presence of CCR2 in the affected tissue might indicate the action of other CCR2 ligand(s). Alternatively, the migration of CCR2-bearing cells into CNS might be a bystander phenomenon.

Compelling evidence has shown that IP-10, MIP-1α, and RANTES are potent factors that attract Th1 T cells into sites of inflammation (65–70). Further, Th1/Th2 T cells have been recently reported to differentially secrete RANTES, lymphotactin, and TCA-3, respectively (71). Enhanced expression of IP-10, MIP-1α, and RANTES in MCP-1$^{+/+}$ EAE CNS tissue and undetectable TCA-3 expression in either MCP-1$^{+/+}$ or MCP-1$^{-/-}$ support the notion that immune reactions within the CNS during EAE attacks are Th1 biased and such responses are more pronounced in mice with an intact MCP-1 gene.

Anti-MOG Ig Isotypes in MCP-1$^{-/-}$ and Wild-type Mice. Sera from MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice with EAE were analyzed for total IgG, IgG1, and IgG2a Abs against the immunizing MOG35–55 peptide. Wild-type and MCP-1–deficient mice produced similar amounts of total MOG-specific IgG (Fig. 10). Despite the disparity in clinical severity between wild-type and MCP-1–null mice, this finding was not unexpected, given the results of experiments using B cell–deficient mice, which showed that Ig does not play an important pathogenic role in MOG35–55 peptide-induced EAE in B6 mice (48).

However, Ig isotype analyses differentiated the wild-type and MCP-1–deficient mice. Levels of anti-MOG35–55 IgG1 Abs in wild-type controls remained low from day 14 pi through day 60 pi (Fig. 10) and were not elevated at intermediate time points (data not shown). In contrast, significantly higher levels of anti-MOG35–55 IgG1 Abs were evident in MCP-1$^{-/-}$ mice (Fig. 10). Levels of anti-MOG35–55 IgG2a Abs showed a trend towards elevated levels in wild-type controls on day 14 pi, whereas no difference between wild-type and MCP-1–null mice was found on day 60 pi (Fig. 10).

Taken together, these results suggest that a polarized MOG35–55–induced Th1 immune response in wild-type mice leads to a suppressed Th2 response, characterized by undetectable IL-4, lower levels of IL-10, lower levels of anti-MOG35–55 IgG1, and higher levels of IgG2a. In this model, the absence of MCP-1 results in a shift towards a Th2-biased response, with reduced production of IFN-γ, enhanced secretion of IL-10, and higher levels of IgG1.

Discussion

We and others have previously shown that MCP-1 was markedly elevated in the CNS of SJL and B6 mice with EAE (33, 35) and levels of MCP-1 expression correlated with the severity of relapsing EAE (72). Anti–MCP-1 Abs blocked relapses of EAE (36). CNS MCP-1 is largely produced by parenchymal astrocytes (34). MCP-1 expression by astrocytes in MS brain lesions has also been convincingly documented (40–42).

However, these studies did not establish a primary role for MCP-1 in disease pathogenesis. In recent definitive studies of EAE using CCR2-deficient mice (37, 38), the relevant ligand for the deleted receptor was not defined. Moreover, MCP-1 exhibits attributes that argue for a role in restraining autoimmune demyelination. In particular, MCP-1 exerts a direct or indirect (via IL-4) impact on Th2 T cell development (11). Further, the presence of MCP-1 in in vitro cell culture systems decreased the encephalitogenic potential of T cells directed to PLP139–151 (36). NK cells that inhibited the encephalitogenic potential of autoaggressive T cells in DA rats produced high levels of MCP-1 in vitro (73). The role(s) of MCP-1 in the pathogenesis and development of EAE (MS) has therefore been uncertain. Using gene-targeted mice, we demonstrate that lack of MCP-1 delays the onset of EAE and ameliorates its severity, by reducing the accumulation of inflammatory leukocytes within CNS. This phenotype was associated with impaired MOG35–55–specific Th1 immune responses.

Impaired macrophage recruitment into the CNS, as indicated by reduced total number of cells and percentage of CD11b$^+$CD45^{++}CD4$^-$ cells recovered from CNS in MCP-1–deficient mice in our study, is consistent with the reduction of macrophages in MCP-1$^{-/-}$ mice in contact hypersensitivity responses (2), in kidney and lung lesions of MCP-1$^{-/-}$ MRL-*Fas^{lpr}* mice (74), in aortic walls of MCP-1 and low density lipoprotein receptor double-deficient mice (75), and in atherosclerosis plaques from MCP-1$^{-/-}$ mice that overexpress apolipoprotein B (76).

Compelling evidence suggests that macrophages and their products can be detrimental in EAE and human MS. Expression of MHC class II is markedly elevated in EAE and MS (77), costimulatory molecules such as CD80, CD86 expressed mainly by macrophages have been demonstrated in MS lesions (78), and blockade of CD28/CD80, CD86 pathway prevents epitope spreading and
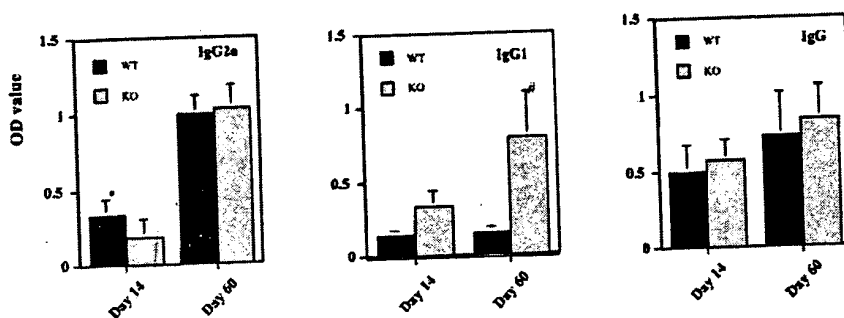

Figure 10. Serum concentrations of anti-MOG35–55 IgG, IgG1, and IgG2a Abs in MCP-1$^{-/-}$ and MCP-1$^{+/+}$ littermate controls after immunization with MOG35–55 plus pertussis toxin (500 ng/injection). Indicated are days pi when sera were collected. Data are expressed as mean ± SD, $n = 6$, in each group. *$P = 0.08$, #$P < 0.01$. WT, wild-type; KO, knockout.

clinical relapses of EAE (79). B7-1/B7-2$^{-/-}$ mice are resistant to EAE induction (47). Similarly, blocking of interactions between CD40 on macrophages and CD40L on T cells has been shown to effectively prevent EAE (80). Products of macrophages like TNF-α, IFN-γ, and nitric oxide have also been demonstrated to be critical in the effector phase of EAE (81, 82). Macrophage depletion inhibits the induction of EAE (83). The absence of clinical EAE in MCP-1$^{-/-}$ recipients of wild-type encephalitogenic T cells further indicates the importance of CNS MCP-1 expression in recruiting macrophages to the CNS. We propose that it is the failure to recruit significant number of macrophages into CNS that constitutes the principal mechanism for resistance to EAE induction in MCP-1–deficient mice. Based on these studies, we cannot exclude the possibility that MCP-1 may directly alter trafficking pattern of dendritic cells in periphery or CNS, expression of costimulators, inflammatory cytokines, and adhesion molecules. Further studies are underway to address these issues.

As several studies have shown that MCP-1 is a critical factor for T cell commitment to the Th2 phenotype, we did not anticipate that MCP-1 gene disruption would result in reduced MOG35–55–specific Th1 immune response in these EAE experiments. Our results show that MOG35–55–specific MCP-1$^{-/-}$ T cells secreted a large amount of IFN-γ, although less than MCP-1$^{+/+}$ T cells, but undetectable levels of IL-4. In view of the reciprocal regulation between IFN-γ and IL-10, the enhanced in vitro secretion of IL-10 by MCP-1$^{-/-}$ T cells might be secondary to reduced levels of IFN-γ. However, increased expression of IL-10 was not observed in vivo as demonstrated by the equal amount of IL-10 transcripts in MCP-1$^{+/+}$ and MCP-1$^{-/-}$ EAE CNS tissue. The fact that MOG35–55–reactive MCP-1$^{-/-}$ T cells mediated severe EAE in wild-type recipient mice in the passive transfer EAE model further suggests that they were Th1 polarized. An explanation for the equal encephalitogenic capacity of MCP-1$^{-/-}$ T cells compared with MCP-1$^{+/+}$ T cells could be that the defective IFN-γ production by MCP-1$^{-/-}$ T cells might be corrected by the presence of IL-12 in the cell culture system (84). Alternatively, IFN-γ concentrations beyond a threshold may be dispensable for encephalitogenic potential. T cells from MCP-1–null mice were shown to produce lower levels of IFN-γ in vitro upon MOG35–55 restimulation in our studies, and MCP-1$^{-/-}$ splenocytes secreted ~50% less IFN-γ when restimulated with *Schistosoma mansoni* eggs in vitro (2), implying a role for MCP-1 in maximal expression of this cytokine under some circumstances. On the premise that MCP-1 is the major ligand for CCR2 in this model, our results are consistent with what has been recently reported in CCR2-deficient mice (38). Thus, a dual function is suggested for MCP-1 in regulating T cell immune responses: promoting Th2 immune responses in certain circumstance while facilitating Th1 responses in others. Such difference is not uncommon when molecules were tested in different animal strains, disease models, and using different immunogens.

We propose that the role of MCP-1 in EAE became manifest because of the extreme Th1 polarization implicated in this model. The impaired ability to mount Th2 responses was not relevant in these experiments because the disease was severely attenuated by the reduction of macrophage recruitment to the CNS. Such reduction of macrophage reaction might subsequently result in reduced Th1 immune responses. Remarkably, in the absence of recruited macrophages, highly polarized Th1 cells became unable to express the Th1 effector program, most clearly demonstrated by decreased circulating and CNS IFN-γ and failure to elicit EAE in MCP-1–deficient mice by MOG-primed MCP-1$^{+/+}$ encephalitogenic T cells.

Taken in the context of recent reports (37, 38), our results indicate that the MCP-1/CCR2 ligand/receptor pair is critical for the expression of EAE in mice. In turn, these findings motivate a continuing effort to characterize the function of this multipotential chemokine in human disease.

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